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Stress, sexual signaling, and alternative mating tactics in male green treefrogs, Hyla cinerea

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STRESS, SEXUAL SIGNALING, AND ALTERNATIVE MATING TACTICS IN MALE GREEN TREEFROGS, *HYLA CINEREA*

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ABSTRACT

This thesis examines how the endocrine stress response mediates the expression of courtship signals and alternative mating tactics in male green treefrogs, *Hyla cinerea*. Males of this species produce acoustic signals that stimulate production of the stress hormone corticosterone (CORT) in vocal competitors, suggesting a role of CORT in intrasexual competition. I predicted that modulation of CORT level during intraspecific contests plays a pivotal role in regulating the expression of male courtship signals and behavior. Consistent with this prediction, CORT administration to calling males, simulating CORT production during male vocal contests, decreased attractiveness by compromising energetic investment in vocalization, increasing aggression, and increasing the probability that a male adopts an alternative non-calling 'satellite' mating tactic. These results suggest that acoustic stimulation of CORT production functions in an armament capacity by compromising the reproductive success of rival males. Secondly, I examined the extent to which CORT and androgen levels, vocal attractiveness, body size, condition, and plasma glucose levels predicted the adoption of satellite behavior in calling males exposed to broadcast advertisement calls (simulating a challenge by another male). Males that adopted satellite behavior in response to broadcast advertisement calls were vocally less attractive, smaller, and in poorer body condition than males that called, but males did not differ in CORT or androgen levels. Lastly, I examined the role of CORT and androgens in male mating behavior by performing a satellite-host manipulation experiment in which I removed the calling host male from the satellite-caller association and observed whether satellite males began to call or continued to exhibit non-calling behavior. Results indicated that satellites were smaller and in poorer body

condition and possessed higher CORT and lower androgen levels relative to host males. Moreover, satellites that did not call subsequent to removal of the calling host male had lower androgen levels relative to host males and satellite males that began to call. Together, my results indicate that circulating CORT level is a target of intra- and intersexual selection and that variation in CORT and androgens levels among males in natural choruses can be a cause or consequence of mating tactic expression.

DEDICATION

I would like to dedicate this research to my family, friends, and colleagues whose encouragement and praise made it possible for me to complete this project.

LIST OF ABBREVIATIONS

HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
CORT	Corticosterone
EHV	Energetics-Hormone Vocalization model
STI	Simulated territorial intrusion
SIL	Snout-ischial length
DHT	Dihydrotestosterone
T	Testosterone
AMTs	Alternative mating tactics

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CHAPTER ONE

THE ACUTE ENDOCRINE STRESS RESPONSE IS A TARGET OF INTRA- AND INTERSEXUAL SELECTION IN GREEN TREEFROGS

Abstract: Whether male courtship signals are reliable indicators of a male's ability to cope with and/or avoid stress is controversial but central to understanding the role of stress in sexual selection. Here, I examine how acute elevations in circulating stress hormones (i.e., glucocorticoids) alter calling behavior in male green treefrogs, *Hyla cinerea*. Males of this species produce acoustic signals that stimulate stress hormone production in rival males competing for females. Given that elevated glucocorticoids characteristically mediate increased investment in self-maintenance over reproduction, I hypothesized that acoustic stimulation of stress hormone production among competing males functions in an armament capacity by decreasing investment in courtship behavior and, thus, attractiveness to females. Consistent with this hypothesis, I show that intraperitoneal injections of corticosterone (CORT; the primary glucocorticoid in amphibians) to calling males rapidly (< 30 min) decreased calling effort, primarily via a reduction in call duration. CORT injections also increased the probability that males produced aggressive acoustic signals, which are unattractive to females. Elevated CORT eventually (< 65 min) caused males to abandon calling behavior and increased the probability of alternative non-calling "satellite" mating tactics. These CORT-mediated effects occurred independently of any detectable change in circulating androgen levels and were most prominent

during simulated territorial intrusions (e.g., broadcast advertisement calls), indicating that stress-mediated effects are context dependent. Together, these results indicate that the acute stress response is a target of both intra- and intersexual selection.

Introduction

The acute endocrine stress response plays an integral role in mediating trade-offs between self-maintenance and current reproduction (Sinervo and Svensson, 1998; Wingfield et al., 1998; Cote et al., 2006; Breuner et al., 2008; Bonier et al., 2009; Hau eta al., 2010; Crespi et al., 2013; Patterson et al., 2014). For example, up-regulation of the hypothalamic-pituitary-adrenal (HPA) axis in response to environmental stressors (i.e., weather, predation, resource availability, social interactions) stimulates the production of glucocorticoids that mediate increased allocation of energy towards behaviors that promote survivorship over reproduction (Wingfield et al., 1998). The adaptive significance of the acute stress response is that it increases survivorship and postpones reproduction until more suitable environmental conditions arise (concepts of the "emergency life-history stage" hypothesis; Wingfield et al., 1998). Individuals often vary in acute glucocorticoid responsiveness (Littin and Cockrem, 2001; Cockrem and Silverin, 2002; Guimont and Wynne-Edwards, 2006; Cockrem, 2007; 2013) and such variation often predicts behavioral responses to the psychosocial environment (Koolhaas et al., 1999; Blas et al., 2007; Cockrem 2007; 2013). For instance, low glucocorticoid responders often behave more audaciously during social interactions (*reviewed* by Blas et al., 2007) and may thus invest more in current reproduction at the cost of survival during environmental challenges. Presumably, the trade-off between survival and reproduction results in equal fitness among individuals exhibiting differences in glucocorticoid responsiveness (Breuner et al., 2008).

Much of what is known regarding the relationship between acute stress responsiveness and fitness stems from examination of performance measures related to survivorship (e.g., growth and developmental rates, foraging behavior, immunocompetence, territoriality; *reviewed* by Breuner et al., 2008) or reproductive output (i.e., fecundity; Contreras-Sanchez et al., 1998; Patterson et al., 2014), but how the acute stress-fitness framework unfolds in the context of sexual selection is poorly understood. For example, few studies have explored the effects of acute elevations in glucocorticoids on sexual signals (Leary et al., 2006a; 2006b) despite evidence indicating that increased acute stress responsiveness potentially decreases investment in reproductive behavior (Wingfield et al., 1998; Breuner et al., 2008; Bonier et al., 2009). Such effects may be particularly important in the context of intrasexual selection because glucocorticoid levels are often modulated by agonistic interactions among males (*reviewed* by Harding, 1981; Creel, 2001; DeVries et al., 2003; Sapolsky, 2005; Creel et al., 2013). For instance, winner-loser contest studies have demonstrated that aggressive interactions can stimulate the production of glucocorticoids in male competitors, with contest losers often showing significantly higher glucocorticoid responsiveness (Bronson and Eleftheriou, 1964; Louch and Higginbotham, 1967; Manogue et al., 1975; Leary, 2014). Given the potential negative effects of high glucocorticoids on reproductive behavior (Wingfield et al., 1998; Breuner et al., 2008; Bonier et al., 2009), variation in glucocorticoid responsiveness during social challenges with rival males may be a critical determinant of male mating success. Here, I examine the "acute stress-fitness" framework in the context of sexual selection to determine how elevations in circulating glucocorticoids, resulting from agonistic male contests, can influence male courtship signals and behavior.

Stress and male-male competition

Male-male competition often involves contests in which males attempt to induce rival males to withdraw, thereby conferring increased opportunities for mate acquisition (Andersson, 1994). Numerous studies have demonstrated that competitive asymmetries in agonistic contests can be influenced by variation in glucocorticoid responsiveness among males (*reviewed* by Harding, 1984; Creel, 2001; DeVries et al., 2003; Creel et al., 2013). Prominent examples include species that establish social hierarchies where dominant males monopolize access to females as well as other resources (e.g., olive baboons, *Papio anubis*, Sapolsky, 1983; African wild dogs, *Lycoan pictus*, Creel et al., 1997; rainbow trout, *Oncorhynchus mykiss*, Øverli et al., 2000). For example, losers of agonistic contests that occur during hierarchical establishment in mice generally exhibit increased stress responsiveness relative to winners (Bronson and Eleftheriou, 1964; Louch and Higginbotham, 1967; Manogue, 1975). These studies paved the way for the "subordinance is stressful" hypothesis (*reviewed* by Creel, 2001) with the notion that physiological suppression of subordinate males is a form of intrasexual competition.

More recently, a plethora of studies have explored the association between stress and social rank in dominance hierarchies with a primary focus on baseline levels of glucocorticoids (e.g., levels when non-stressed). Baseline levels of glucocorticoids can be either positively or negatively correlated with social rank (*reviewed* by Creel, 2001; DeVries et al., 2003; Gilmour et al., 2005; Sapolsky, 2005; Creel et al., 2013) with the direction of the correlation being influenced by hierarchical type (i.e., despotic or linear), hierarchical stability, stress-coping strategies, and the style of breeding system (i.e., cooperative or non-cooperative; Sapolsky, 2005). For instance, subordinance is generally associated with high baseline levels of circulating glucocorticoids, particularly among non-cooperative breeders (Louch and Higginbotham, 1967;

Popova and Naumenko, 1972; Manogue et al., 1975; Coe et al., 1979; Rohwer and Wingfield, 1981; Schwabl et al., 1988; Virgin and Sapolsky, 1997). In many cooperative breeders, however, dominance is generally associated with high baseline levels of glucocorticoids (Schoech et al., 1991; Creel et al., 1992; 1996; 1997; Smith and French, 1997, Cavigelli, 1999).

The relationship between stress-induced levels of glucocorticoids and dominance or subordinance may also be influenced by hierarchical stability (*reviewed* by DeVries et al., 2003). During times of social stability in olive baboons, for example, dominant males exhibit lower baseline levels of glucocorticoids relative to subordinates, but also exhibit higher glucocorticoid responsiveness (Sapolsky, 2005). Conversely, subordinate salmonid fish often exhibit higher glucocorticoid responsiveness compared to dominant males (*reviewed* by Gilmour et al., 2005) and, moreover, the magnitude of the stress response appears to be heritable (Pottinger and Carrick, 2001). For example, male rainbow trout bred for low glucocorticoid responsiveness are dominant over high glucocorticoid responsive males during rank-order fights (Pottinger and Carrick, 2001), suggesting that variation in glucocorticoid responsiveness predicts contest outcomes. This may be exploited by dominant males during hierarchical establishment because eliciting glucocorticoid production in subordinate males can potentially suppress investment in reproduction.

Stress and elaborate male traits

Dominance hierarchies are particularly extreme examples of intrasexual competition because aggressive encounters may escalate to physical combat and the social order can inherently limit the access of subordinate males to females, especially in "despotic" systems where reproduction is highly skewed towards a dominant male (see Sapolsky, 2005; Creel et al.,

2013). However, in species where males perform courtship displays and females choose males based on attractiveness of elaborate male traits (i.e., lekking species), males may be expected to invest in traits which effectively compromise the competitive ability of rival males by decreasing rival male attractiveness. Considerable emphasis has been placed on understanding the role of acute fluctuations in circulating levels of glucocorticoids on the expression and magnitude of elaborate male traits (Buchanan, 2000; Husak and Moore, 2008; Leary and Knapp, 2014; Moore et al., 2015). The working hypothesis is that elevations in glucocorticoids, through their role in gluconeogenesis, may be required to meet the metabolic demands of costly sexual traits, with moderate elevations enhancing the magnitude of trait elaboration and high circulating levels of glucocorticoids negatively affecting trait elaboration (Saino et al., 2002; Adkins-Regan, 2005; Leary et al., 2006a; 2006b; Roulin et al, 2008; Bortolotti et al., 2009; *reviewed* by Leary and Knapp, 2014). Glucocorticoids may directly affect the central nervous system to alter the magnitude of trait elaboration or could be linked to immunosuppression and/or suppression of the hypothalamic-pituitary-gonadal (HPG) axis and androgen production (*reviewed* by Leary and Knapp, 2014).

To date, much of what is known regarding the association between glucocorticoids and elaboration of sexual traits is correlative (see Moore et al., 2015) and provides mixed support for a link between glucocorticoid level and the elaboration of sexual traits. For example, glucocorticoids can be negatively correlated (Douglas et al., 2009; Henderson et al., 2013; Kennedy et al., 2013; Merrill et al., 2014), or uncorrelated with male sexual traits (Parker et al., 2002; Setchell et al., 2010; Jenkins et al., 2013). Furthermore, several studies in avian species have explored the organizational (or permanent) role of developmental stress on song complexity in male song birds (Buchanan et al., 2003; Spencer et al., 2003; 2004; MacDougall-Shackleton et

al., 2009; Schmidt et al., 2012). However, few studies have explored activational (or ephemeral) effects of acute elevations in glucocorticoids on the magnitude of trait elaboration (Leary et al., 2006a; 2006b). Moreover, in a cross species meta-analysis, there was no evidence that glucocorticoid level and other measures of stress (i.e., oxidative stress and heterophil-to-lymphocyte ratio) predicted the expression or magnitude of elaborate male traits; however, there was evidence suggesting that females prefer males with low levels of glucocorticoids (Moore et al., 2015). Thus, the role of glucocorticoids in the mediating the expression and magnitude of elaborate male traits is poorly understood.

Stress and sexual signaling in male green treefrogs

Here, I examine the role of glucocorticoids in the context of intra- and intersexual selection in green treefrogs, *Hyla cinerea*. Males of this species produce acoustic signals that increase corticosterone (CORT) and decrease androgens in signal receivers (Leary, 2014). This provides an opportunity to investigate the role of glucocorticoids in male-male competition of a lekking species in which females choose males based on vocal attractiveness.

Male *H. cinerea* primarily produce two types of acoustic signals: advertisement calls and aggressive calls (Gerhardt and Huber, 2002). Advertisement calls are used to attract females and deter males (Wells, 1977) and are characteristically unpulsed (i.e., not amplitude-modulated) with the exception of a pulsed prefix (~ 24 ms pulse duration) at the onset of the call (Fig. 1A). Conversely, aggressive calls are highly amplitude modulated throughout and are exclusively produced during male-male agonistic interactions (Fig. 1B). While advertisement calls often elicit elevations in circulating levels of androgens in nearby male signalers (Burmeister and Wilczynski, 2000), aggressive calls are highly effective at increasing CORT and decreasing

androgens in signal receivers (Leary, 2014). The primary goal of the present study was to examine whether acoustic modulation of glucocorticoid production during male contests alters the attractiveness of male acoustic signals.

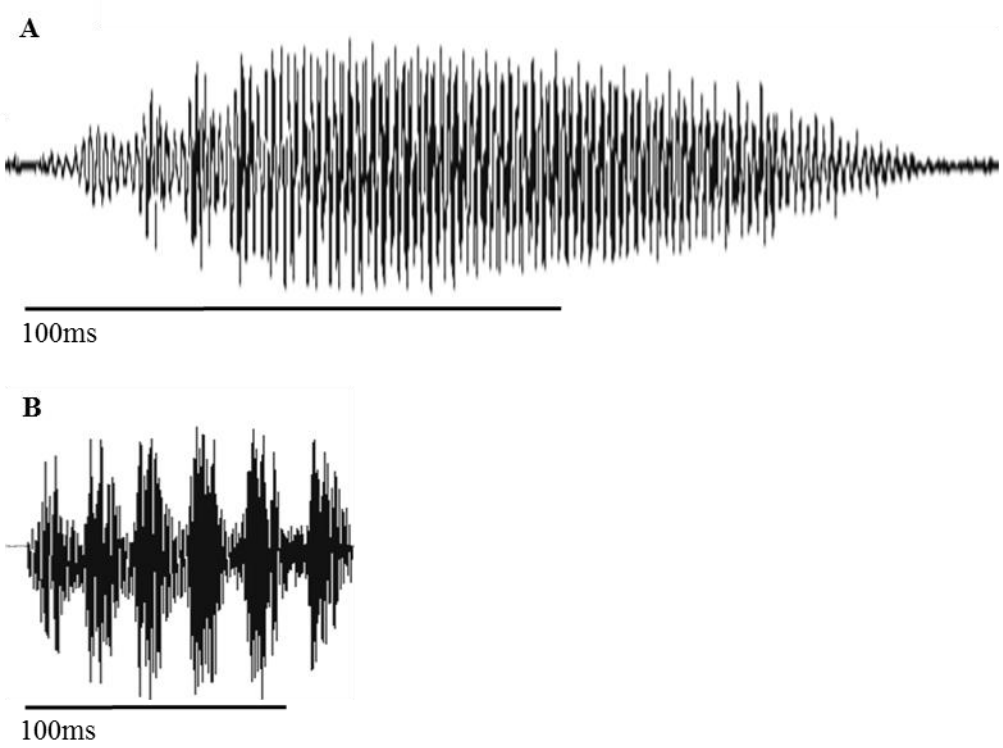

Figure 1. (A) Waveform representation of an advertisement call produced by male *H. cinerea*. (B) Waveform representation of an aggressive call which, unlike the advertisement call, is highly amplitude modulated.

Advertisement calls of male *H. cinerea* consist of both spectral and temporal properties that are important for mate choice by females (Gerhardt and Huber, 2002). The advertisement calls are broadband signals possessing two dominant carrier frequencies (Gerhardt et al., 1990; Gerhardt and Huber, 2002). Advertisement calls typically possess a low peak frequency of approximately 900 Hz and a high peak frequency of 3000 Hz (Gerhardt et al., 1990), although average values can differ between populations. Choice-based experiments have shown that

females generally prefer frequency bands that approximate the mean of the local population, though preferences can be reduced with increasing sound intensity (e.g. sound pressure level, SPL; Gerhardt, 1982; 1987; 1991) suggesting that frequency preferences may not be particularly strong (*reviewed* by Gerhardt and Huber, 2002). Carrier frequencies are often correlated with body size (Davies and Halliday, 1978) because body size is correlated with the size of sound producing structures (i.e., trunk muscles and larynx; *reviewed* by Gerhardt and Huber, 2002).

In contrast, temporal properties, such as call duration and call rate, are under strong directional selection because of female preference for higher-than-average values that are indicative of high energetic investment in vocalization (Gerhardt and Huber, 2002). Female *H. cinerea* generally prefer calls of long duration produced at high rates (Gerhardt and Huber, 2002). Call duration and rate are often used to calculate a male's vocal effort, or the proportion of time spent calling (Pough et al., 1992; Gerhardt and Huber, 2002). Vocal effort is generally considered a proxy for energetic investment in vocalization because vocal effort is positively correlated with aerobic metabolic rate and contributes to overall acoustical energy (see Taigen and Wells, 1985; Pough et al., 1992; Prestwich, 1994). Vocal effort is often influenced by rival male calling activity (Pough et al., 1992); for instance, males will increase call duration and call rate in response to rival male signals (*reviewed* Gerhardt and Huber, 2002). The Energetics-Hormone Vocalization (EHV) model predicts that elevations in vocal effort are accompanied by elevations in CORT which mobilize energy reserves to meet the metabolic demands of increased signaling (Emerson, 2001). However, high CORT is predicted to suppress circulating levels of androgens that decrease vocal effort (Emerson, 2001) suggesting that variation in CORT level can alter vocal attractiveness.

Males producing vocalizations that are unattractive to females may use an alternative non-calling "satellite" mating tactic to maximize mating success. Satellite males crouch near calling males and attempt to intercept females attracted to the calling host's vocalizations (Sullivan, 1982). Tactic switching is known to occur frequently within and across nights of chorus activity, suggesting that variation in mating tactic expression is condition-dependent (Gross, 1996). Satellite *H. cinerea* males are often smaller than host males and vocally less attractive (Humfeld, 2008; see Chapter 3). While body size can influence various call attributes in anurans (i.e., dominant carrier frequencies; Davies and Halliday, 1978), the size of male *H. cinerea* can also affect the magnitude of glucocorticoid secretion during agonistic interactions with vocal competitors, with smaller males exhibiting greater up-regulation of the HPA axis during male vocal contests (Leary, 2014). The EHV model predicts that the cessation of calling behavior is a consequence of negative effects of elevated CORT levels on gonadal steroids. In support of this hypothesis, satellite *H. cinerea* have higher circulating levels of CORT and lower levels of androgens compared to calling males (Leary and Harris, 2013; see Chapter 3). However, it has not been established whether adoption of the satellite mating tactic by male *H. cinerea* is causally related to elevations in circulating CORT levels.

The current study investigates the effects of elevated CORT on vocal attractiveness and calling behavior of male *H. cinerea*. The objective was to determine what males gain by eliciting CORT production in rival males during vocal contests. Considering the EHV model (Emerson, 2001) and the predicted negative effects of high CORT on elaborate male traits (Buchanan, 2000; Husak and Moore, 2008; Leary and Knapp, 2014; Moore et al., 2015), I hypothesized that acoustic stimulation of CORT production functions in an armament capacity by compromising male vocal attractiveness and increasing the probability of satellite tactic expression.

Methods

Pre-treatment

Male *H. cinerea* were studied in natural choruses at the University of Mississippi (UM) Field Station (Lafayette County, MS, USA), during peak hours of chorus activity (~2000-2200 hour) over the course of the 2013 and 2015 breeding seasons (April – July each year). Experiments were first performed to assess the suitability of experimental subjects for hormone (i.e., CORT) injection procedures. This involved locating pairs of calling males that were at least 7 m from another calling male and approximately the same size (based on initial visual assessment). These selection criteria helped to control for the potential effects of the social-acoustic environment on vocal behavior and/or hormone levels, and possible size-related differences in responses to experimental procedures. A series of approximately 30 calls were then recorded from both males using a Marantz PMD 222 cassette recorder equipped with a Sennheiser directional condenser microphone (Model ME-66). Subsequently, an advertisement call stimulus was broadcast to calling males, simulating a territorial intrusion, while vocalizations of males were continually recorded. Broadcast advertisement calls used during a stimulated territorial intrusion (STI) consisted of a single isolated call with the mean call attributes of the study population (e.g., dominant carrier frequencies = 800 and 2600 Hz, call duration = 189 ms, intercall duration = 612 ms) based on analysis of approximately 500 individuals (Davis and Leary, 2015). The advertisement call stimulus was repeatedly broadcast in a continuous loop from a hand-held speaker (Marantz PMD 222 recorder) for a two-minute focal period from a distance of 30 cm and at an amplitude of 90 dB SPL (measured with a hand-held sound pressure level meter, Ivie Technologies Inc., model IE-45, fast weighting setting) that reflected the amplitude of natural calls for this species (Humfeld, 2013). This procedure was

repeated twice prior to treatment to ensure that males continued to call in response to the broadcast stimulus. The experiment was terminated if one of the paired males stopped calling in response to one of the broadcast trials.

Post-treatment

Individuals that continued to call in response to the broadcast stimulus in the two trials were then administered either 100µL saline (n=12), or 8µg CORT in a 100µL saline vehicle (n=12). All males resumed calling behavior within 5 min following injection and were allowed to call for 10 min before a series of post-injection vocalizations were recorded. Vocalizations were then recorded post-treatment approximately every 10 min in the absence and presence of a STI. This was repeated every 10 min for up to 75 min or until at least one male in each pair stopped vocalizing in response to the broadcast stimulus, whichever came first. At the end of the experiment, blood samples were rapidly obtained (< 2 min) using cardiac puncture with a heparinized needle. Snout-ischial length (SIL) and weight were measured and males were marked on the venter using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL, USA) for future identification after release at the site of capture. Blood samples were stored on ice until they were brought to the laboratory (~2 h later) where they were immediately centrifuged for 12 min at 2200 rpm so that the plasma could be removed. Plasma samples were then stored at -20ºC until dihydrotestosterone (DHT), testosterone (T), and corticosterone (CORT) levels were quantified using column chromatography and radioimmunoassay (described below).

Validation of CORT injections

Pre-injection blood samples were not acquired from experimental subjects because of the potential effects of bleeding procedures on calling behavior. Hence, without knowledge of an individual's hormone levels prior to treatment, the magnitude of the increase in CORT level and/or potential reduction in androgens over the course of the experiments could not be assessed. To address these issues, I performed injection procedures on additional calling males (n = 6 for saline injections; n = 15 for CORT injections) in natural choruses and in the absence of STIs but acquired both pre- and post-injection blood samples (approximately 1 h post-injection).

Call Analysis

Calls were analyzed using Raven Pro acoustics software (Cornell Bioacoustics Laboratory). I examined call parameters known to be important for mate selection in this species (Gerhardt, 1974; 1978b; 1982; 1987; 1991; Gerhardt et al., 1990; Gerhardt and Huber, 2002; Humfeld, 2008) from spectrograms, spectra, and waveforms. I measured call duration (ms), intercall duration (ms), and call rate (calls/min), as well as low and high dominant carrier frequency (kHz) for 10 calls from each male immediately before treatment and every 10 to 15 min post-treatment for up to 75 min. I then calculated vocal effort (i.e., proportion of time spent calling) by dividing call duration by the sum of call duration and intercall duration. The extent of vocal data obtained post-treatment for calling males was dependent on whether vocal abandonment occurred and at what time it occurred during experimentation. I analyzed calls in the absence and presence of a STI at each time point to determine if there was an interaction between hormone treatment (i.e., CORT or saline) and social context (i.e., STI or no STI) on the quality of vocalizations based on known female preferences for calls.

Body condition

Body condition was used as an estimate of energy reserves and was calculated by taking the residual values from a linear regression of the cubed root body weight on SIL divided by SIL (see also Baker, 1992; Leary et al., 2008).

Sample preparation, column chromatography, and radioimmunoassay procedures

Hormone separation and quantification of hormone concentrations followed Leary et al. (2004). Briefly, 30µL of each plasma sample was allocated into a separate extraction tube and incubated overnight with radiolabeled hormone (PerkinElmer, Inc. Hebron, Kentucky). Steroids were then extracted from the plasma using diethyl ether and subsequently dried under nitrogen gas at 40ºC. Dried samples were then resuspended in 10% ethyl acetate in iso-octane and loaded onto diatomaceous earth columns that contained a 3:1 diatomaceous earth:distilled water "glycol trap" and a 1:1 propanediol:ethylene glycol mixture. Mixtures of 10%, 20%, and 52% ethyl acetate in iso-octane were then used to collect dihydrotestosterone (DHT), testosterone (T), and corticosterone (CORT), respectively. Fractions were dried under nitrogen gas and resuspended in phosphate buffered saline containing 0.3% gelatin for use in radioimmunoassay. The T antibody was purchased from Fitzgerald Industries International, Inc. (Acton, Massachusetts) and used to measure both DHT and T. The CORT antibody was purchased from MP Biomedicals, LLC (Solon, Ohio).

Plasma samples were analyzed for circulating DHT, T, and CORT levels in two assays; one that included 24 males sampled once for blood at the end of the experimental procedures and the other including 20 males sampled twice for blood prior to and subsequent to injections. Mean

intra-assay coefficients of variation for DHT, T, and CORT were 8%, 9%, and 4%, respectively, based on 4 standards run with each assay. Inter-assay coefficients of variation for DHT, T, and CORT were 5%, 5%, and 4.6%, respectively.

Statistical analysis

To assess suitability of parametric tests in downstream analyses, I tested for homogeneity of variance and normality for all measured parameters. I first examined whether paired CORT- and saline-injected males differed in size, weight, and/or body condition using one-way analysis of variance (ANOVA). Paired *t*-tests were then used to examine circulating levels of DHT, T, and CORT from males sampled for blood prior to and after injection. An ANOVA was then used to compare circulating levels of androgens and CORT between saline-injected and CORT-injected males sampled only once following treatment.

A mixed model repeated measures ANOVA was then performed for each measured vocal parameter to explore the within-group changes in vocalization over the course of the experiment. Repeated measures ANOVAs were designed using time and social context (i.e., STI or no STI) as within-subject factors, and treatment as the between-subject factor. Mauchly's test of sphericity was utilized to test the repeated measures ANOVA assumption of sphericity for within-subject effects (Mauchly, 1940). If the assumption of sphericity was violated, repeated measure ANOVA results were reported with a Greenhouse-Geisser correction (Greenhouse and Geisser, 1959). A separate within-CORT subjects repeated measures ANOVA was performed on all vocal parameters with SIL as a covariate to investigate whether changes in the vocalization of males after CORT treatment was influenced by the body size of individuals.

Lastly, I employed a Fisher's exact test using non-repeated measures data to determine whether there is an association between CORT administration and the probability of calling abandonment. Next, I determined whether the effects of CORT on calling behavior were influenced by the social-acoustic environment. To test this, I performed a second Fisher's exact test with CORT-injected males with the inclusion of social context. Males that were sampled using repeated measures were not exposed to a STI, but were instead isolated following injection providing an opportunity to test the effects of the environment on the probability of calling abandonment. All statistical analyses were performed using Statview (SAS Institute, Inc.) and SPSS (IBM Software).

Results

Size, weight, and body condition of paired males

There was no evidence that CORT-injected and saline-injected males differed in SIL ($F_{1, 22} = 0.005$, $p = 0.94$; $\eta^2 = 0.000$; Fig. 2A), weight ($F_{1, 22} = 0.007$, $p = 0.93$; $\eta^2 = 0.000$; Fig. 2B), or body condition ($F_{1, 22} = 0.04$, $p = 0.84$; $\eta^2 = 0.002$; Fig. 2C). The range of sizes for males injected with CORT and saline (range: 47 – 62 mm, mean = 55 ± 0.58) spanned much of the range of sizes found in both satellite males (range: 41 – 60 mm, mean = 50 ± 1.00) and calling males (range: 44 – 66 mm, mean = 56 ± 0.40) in natural choruses (Leary and Harris, 2013).

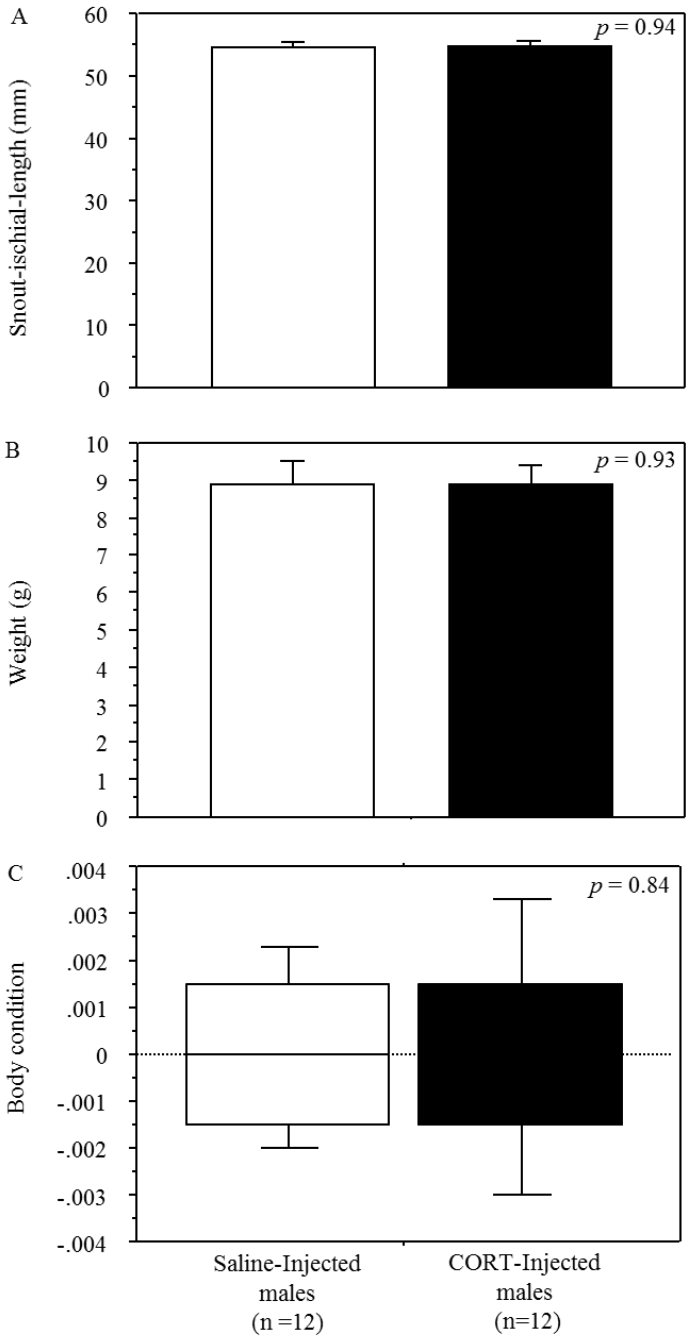

Figure 2. Bar graphs depicting the average (A) body size, (B) weight, and (C) condition of saline-injected and CORT-injected males. Whiskers indicate ±1 SE.

Effects of treatment on circulating hormone levels

CORT administration significantly increased circulating CORT levels in males that were sampled for blood prior to and after CORT injections ($t_{(14)}$ = -8.28, $p < 0.0001$; Cohen's d = 2.14; Fig. 3A). The mean magnitude of the CORT increase was 47.62 (± 5.78 SE) ng/mL, simulating the magnitude of the CORT increase elicited by broadcast calls in playback experiments (*see* Leary, 2014). There was no evidence of a change in circulating levels of DHT ($t_{(14)}$ = -0.59, p = 0.56; Cohen's d = 0.15), or circulating levels of T ($t_{(14)}$ = -0.35, p = 0.73; Cohen's d = 0.10) in CORT-injected individuals sampled prior to and after treatment (Fig. 3A). Saline-injected individuals exhibited no significant change in circulating levels of CORT pre and post-treatment ($t_{(4)}$ = 0.36, p = 0.73; Cohen's d = 0.16; Fig. 3A), nor was there evidence of a change in circulating levels of DHT ($t_{(4)}$ = -1.45, p = 0.22; Cohen's d = 0.64; Fig. 3A), or circulating levels of T ($t_{(4)}$ = -0.92, p = 0.41; Cohen's d = 0.41; Fig. 3A).

The results from males that were only sampled for blood post-treatment were consistent with those obtained from males sampled prior to and after treatment. For example, CORT and saline-injected individuals did not differ in circulating levels of DHT ($F_{1, 22}$ = 0.18, p = 0.38; η^2 = 0.04; Fig. 3B), or circulating levels of T ($F_{1, 22}$ = 0.04, p = 0.85; η^2 = 0.002; Fig. 3B). However, those males injected with CORT had significantly higher levels of circulating CORT relative to saline-injected males ($F_{1, 22}$ = 44.03, $p < 0.0001$; η^2 = 0.67; Fig. 3B). Across all males treated with CORT, circulating CORT levels post-treatment (range: 17.20 – 138.77 ng/ml; mean = 56.17 ± 4.91, n = 28) fell below the upper physiological range of CORT levels found in this species (range of CORT above the 3rd quartile = 6.01 to 170. 67 ng/ml, mean = 21.68 ± 5.60, n = 140).

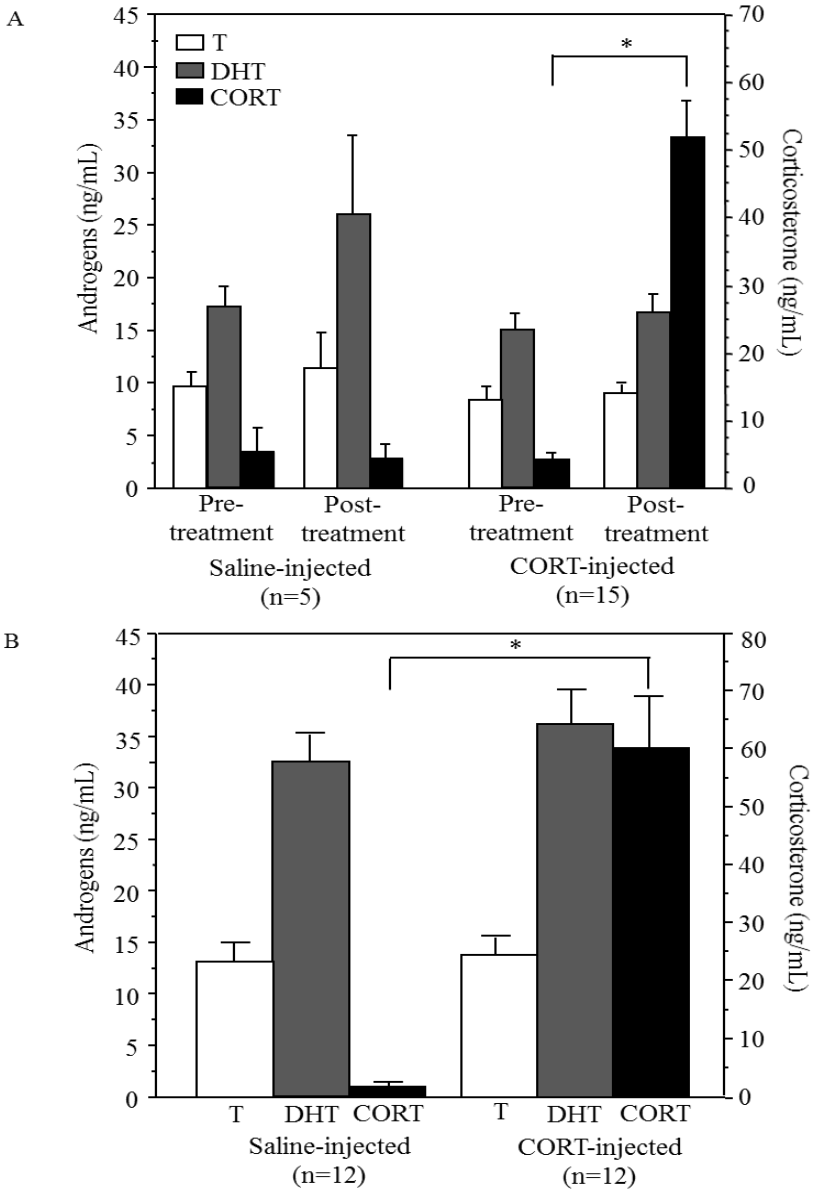

Figure 3. (A) Bar graph depicting circulating levels of testosterone (T), dihydrotestosterone (DHT) and corticosterone (CORT) in male *H. cinerea* before and after treatment with saline or corticosterone. (B) Mean testosterone (T), dihydrotestosterone (DHT), and corticosterone (CORT) levels in male *H. cinerea* after treatment with saline or corticosterone. Bar with asterisk indicates a significant difference between hormone levels. Whiskers indicate ±1 SE.

The effects of CORT on calling behavior

All *p*-values for main and interactive effects from repeated measures ANOVAs for each measured call parameter are provided in Table 1. Note that there was one CORT-injected male

that abandoned calling behavior approximately 7 min post-treatment, which was prior to the onset of recordings for post-treatment vocalizations. Thus, this male and his paired saline-injected male were not included in the vocal analysis.

(a) Call duration

There was not a significant main effect of time on call duration ($F_{2, 40} = 2.67$, $p = 0.08$; $\eta^2 = 0.12$; Table 1). However, there was a significant interaction between time and treatment on call duration ($F_{2, 40} = 3.69$, $p = 0.03$; $\eta^2 = 0.16$; Table 1), with CORT-injected individuals exhibiting a greater decrease in call duration over time compared to saline-injected males (Fig. 4A). Additionally, there was a significant main effect of social context on call duration ($F_{1, 20} = 25.13$, $p < 0.0001$; $\eta^2 = 0.56$ Table 1); males increased the duration of calls in response to STIs (Fig. 4A). However, there was no significant interaction between social context and treatment on call duration ($F_{1, 20} = 1.05$, $p = 0.32$; $\eta^2 = 0.05$; Table 1).

(b) Intercall duration

Mauchly's test indicated that the assumption of sphericity had been violated for the main effect of time ($\chi^2_{(2)} = 9.14$, $p = 0.01$). Therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.72$). The main effect of social context was not tested for the assumption of sphericity as there were only two categorical levels. The corrected results for intercall duration indicated a significant main effect of time on intercall duration ($F_{1.45, 28.95} = 16.92$, $p < 0.0001$; $\eta^2 = 0.46$; Table 1), which gradually increased among all males over the course of the experiment (Fig. 4B). However, there was no significant interaction between time and treatment on intercall duration ($F_{1.45, 28.95} = 0.50$, $p = 0.55$; $\eta^2 = 0.02$; Table 1; Fig. 4B). There

was, however, a main effect of social context on intercall duration ($F_{1, 20} = 6.58$, $p = 0.02$; $\eta^2 = 0.25$; Table 1) with males exhibiting an increase in intercall duration in the presence of a STI (Fig. 4B). However, there was no significant interaction between social context and treatment on intercall duration ($F_{1, 20} = 0.52$, $p = 0.48$; $\eta^2 = 0.03$; Table 1; Fig. 4B).

(c) Call rate

There was not a significant main effect of time on call rate ($F_{2, 40} = 0.24$, $p = 0.79$; $\eta^2 = 0.01$; Table 1), nor was there a significant interaction between time and treatment on call rate ($F_{2, 40} = 0.60$, $p = 0.55$; $\eta^2 = 0.03$; Table 1). However, there was a main effect of social context on call rate ($F_{1, 20} = 12.85$, $p = 0.002$; $\eta^2 = 0.39$; Table 1); males generally decreased the rate at which calls were produced when exposed to STIs (Fig. 4C). The main effect of social context on call rate was primarily a result of the significant interaction between social context and treatment on call rate ($F_{1, 20} = 6.98$, $p = 0.01$; $\eta^2 = 0.26$; Table 1) in which the magnitude of the change in call rate in the presence and absence of STIs was much more pronounced in CORT-injected males compared to saline-injected males (Fig. 4C).

(d) Vocal effort

There was a significant main effect of time on vocal effort ($F_{2, 40} = 15.79$, $p < 0.0001$; $\eta^2 = 0.44$; Table 1) with vocal effort decreasing over time among all males (Fig. 4D). Moreover, there was a significant interaction between time and treatment on vocal effort ($F_{2, 40} = 4.42$, $p = 0.02$; $\eta^2 = 0.18$; Table 1), with CORT-injected males showing a greater decrease in vocal effort over time relative to saline-injected males (Fig. 4D). However, there was not a significant main effect of social context on vocal effort ($F_{1, 20} = 0.33$, $p = 0.57$; $\eta^2 = 0.02$; Table 1), nor was there a

significant interactive effect of social context and treatment on vocal effort ($F_{1, 20} = 0.00$, $p = 1.00$; $\eta^2 = 0.000$; Table 1).

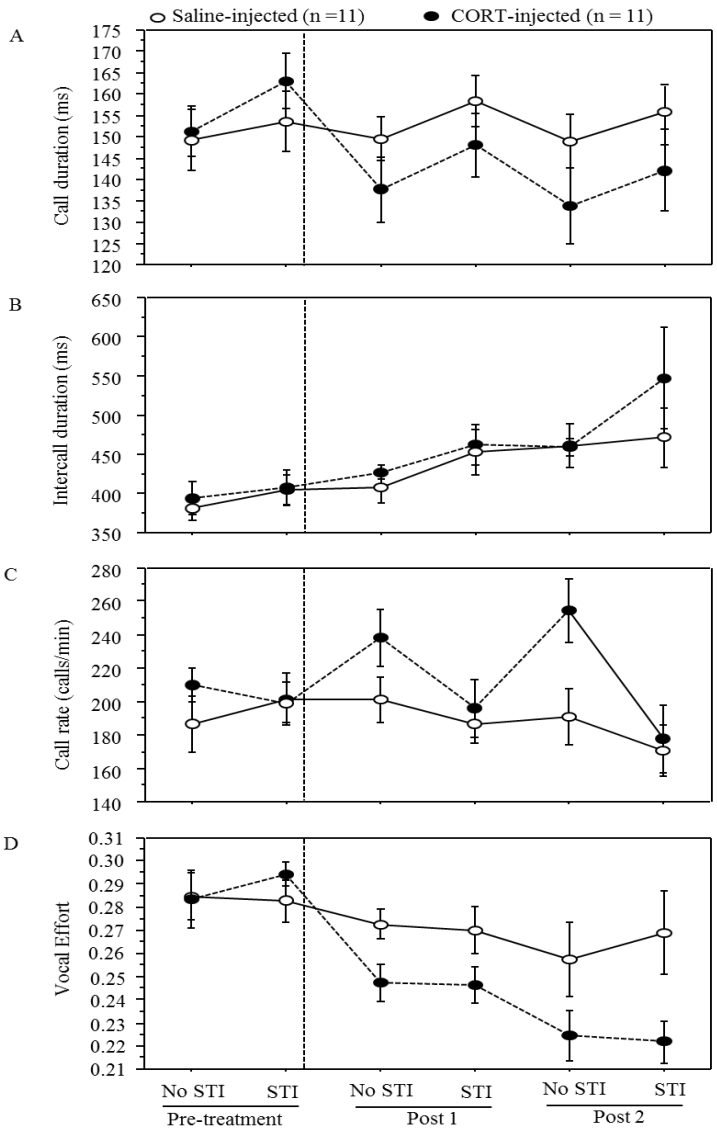

Figure 4. Line graphs showing mean (A) call duration, (B) intercall duration, (C) call rate, and (D) vocal effort prior to treatment and up to 65 min post-treatment and in the presence or absence of a simulated territorial intrusions (STI) for saline and CORT-injected males. The vertical dotted line represents approximate time of injection. The x-axis depicts the sampling period wherein "Post 1" represents a sampling time between 15 and 30 min post-injection, and "Post 2" represents a sampling time between 30 and 65 min post injection. Each point on the graph does not represent calls recorded at the same time post-injection because CORT-injected males stopped calling anywhere between 7 and 65 minutes. Thus, I used the time at which final recordings were obtained from CORT-injected males to designate the post 2 sample period for that pair and then I divided that time in half to represent sample period post 1. Whiskers indicate ±1 SE.

(e) Low-frequency spectral peak

There was not a significant main effect of time on the low-frequency spectral component of advertisement calls ($F_{2, 40} = 0.7$, $p = 0.93$; $\eta^2 = 0.003$; Table 1) indicating that the low peak frequency was relatively constant for all males over the duration of the experiment (Fig. 5A). However, there was a significant interaction between time and treatment on the low peak frequency ($F_{2, 40} = 4.29$, $p = 0.02$; $\eta^2 = 0.18$; Table 1) with CORT-injected individuals exhibiting a decrease in the low peak frequency of their advertisement calls followed by an increase towards the end of the experiment, compared with saline-injected males whose low frequency increased post–treatment, but decreased towards the end of the experiment (Fig. 5A). Furthermore, there was a significant main effect of social context on the low peak frequency ($F_{1, 20} = 4.77$, $p = 0.04$; $\eta^2 = 0.19$; Table 1) with males increasing the low peak frequency in the presence of a STI (Fig. 5A); however, there was no interaction between context and treatment on the low peak frequency ($F_{1, 20} = 0.002$, $p = 0.96$; $\eta^2 = 0.000$; Table 1).

(f) High-frequency spectral peak

For the high-frequency spectral component of the advertisement call, there was no detectible main effect of time ($F_{2, 40} = 0.74$, $p = 0.48$; $\eta^2 = 0.04$; Table 1) or an interaction between time and treatment ($F_{2, 40} = 0.085$, $p = 0.92$; $\eta^2 = 0.004$; Table 1) indicating that the high peak frequency was relatively constant over the duration of the experiment regardless of treatment for both groups (Fig. 5B). However, there was a main effect of social context ($F_{1, 20} = 9.07$, $p = 0.007$; $\eta^2 = 0.31$; Table 1), with males slightly increasing their high peak carrier frequency in the presence of a STI (Fig. 5B). There was no interaction between social context and treatment on high peak frequency ($F_{1, 20} = 2.99$, $p = 0.09$; $\eta^2 = 0.13$; Table 1).

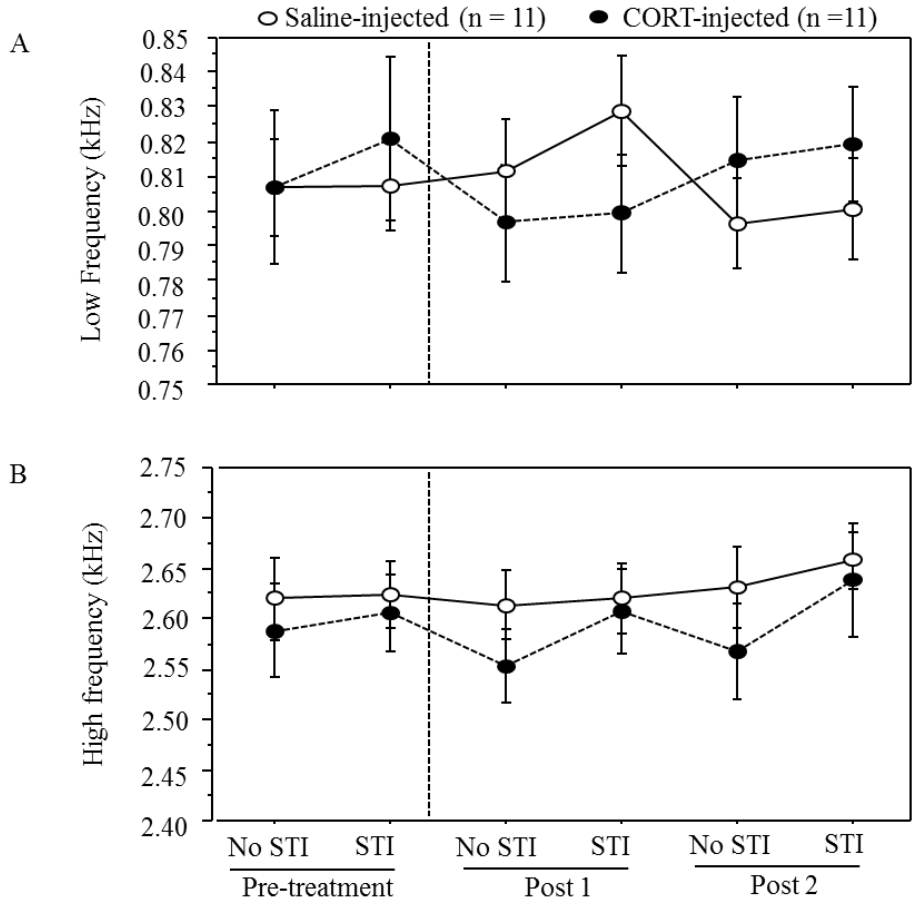

Figure 5. Line graphs showing the mean of the (A) low-frequency and (B) high-frequency spectral peaks of advertisement calls prior to treatment (pre-treatment) and up to 65 min post-treatment and in the presence or absence of a simulated territorial intrusions (STI) for saline and CORT-injected males; The vertical dotted line represents approximate time of injection. The sampling period was designating in the same fashion as those for temporal properties shown in figure 5. Whiskers indicate ±1 SE.

Table. 1. *P* values from within-subject effects of a mixed model repeated measures ANOVA for all acoustic parameters acquired from saline (n=11) and CORT-injected (n=11) male *Hyla cinerea*.

	Within-Subject Effects			
Call Parameter	*p*(time)	*p*(time*treatment)	*p*(context)	*p*(context*treatment)
Call Duration (ms)	0.08	**0.03**	< **0.0001**	0.32
Intercall Duration (ms)	< **0.0001**	0.55	**0.02**	0.48
Call Rate (calls/s)	0.79	0.55	**0.002**	**0.02**
Vocal Effort	< **0.0001**	**0.02**	0.57	1.00
Low Carrier Frequency (kHz)	0.93	**0.02**	**0.04**	0.96
High Carrier Frequency (kHz)	0.48	0.92	**0.007**	0.09
Proportion of aggressive calls	**0.001**	**0.001**	**0.001**	0.06*

Bold indicates significance, alpha at 0.05.
*Indicates marginal significance.

Size effects on responsiveness to CORT-injections

Lastly, I examined whether vocal responsiveness to CORT injections varied with body size. There was no effect of body size on the changes in call duration**,** intercall duration, call rate, calling effort, low carrier frequency, or high carrier frequency within CORT-injected males ($p >$ 0.09 for all parameters) suggesting that males experience similar changes in vocalization regardless of body size.

The effects of CORT on male aggression

During experimentation, there was an observed increase in aggression in CORT-injected males that was unexpected. Thus, I ran a Fisher's exact test to determine whether CORT administration was associated with an increase in the propensity to become aggressive. Of the CORT-injected males, 8 of 12 (67%) increased aggression post-treatment whereas 1 of 12 (8%) saline-injected males increased aggression post-treatment (Fig. 6). There was a statistically significant association between CORT administration and the probability of increased aggression (Fisher's exact test; p =0.005).

To further explore these effects, I ran a mixed model repeated measures ANOVA to examine changes in the proportion of aggressive calls in males injected with either CORT or saline over the course of the experimental procedure. Mauchly's test indicated that the assumption of sphericity had been violated for the main effect of time ($\chi^2_{(2)} = 7.74$, $p = 0.02$). Therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.76$). I found a significant main effect of time on the proportion of aggressive calls ($F_{1.6, 32.6} = 9.19$, $p = 0.001$; $\eta^2 = 0.32$; Table 1) indicating that the proportion of aggressive calls increased over time among all males (Fig. 7).

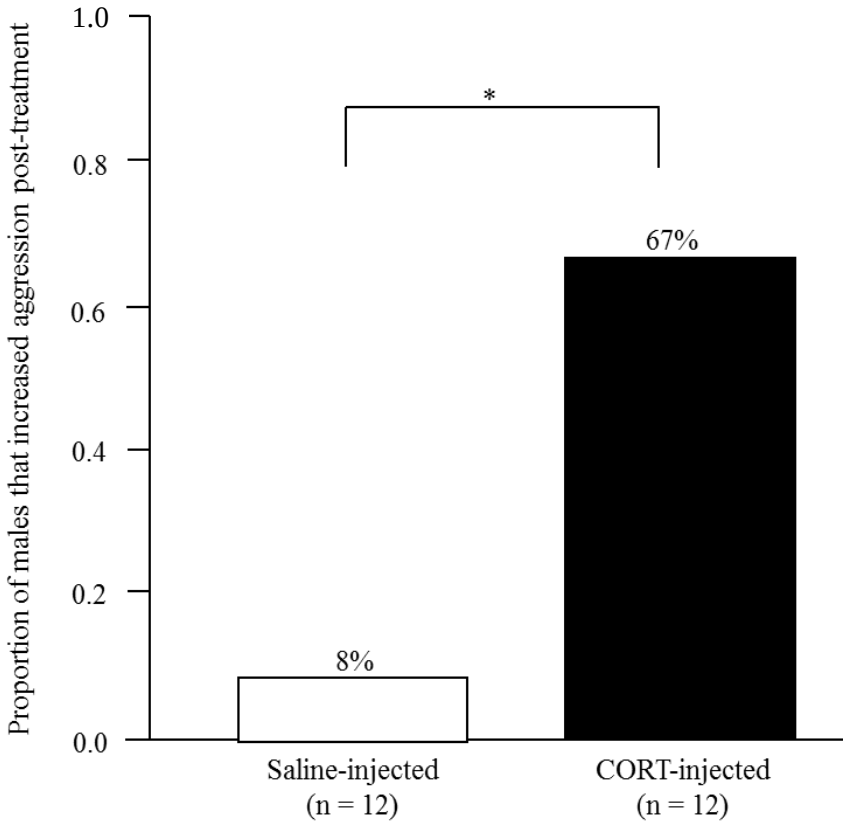

Figure 6. Bar graph indicating the proportion of saline and CORT-injected males that increased aggression subsequent to saline and CORT injections. Bar with asterisk indicates significant difference in the proportion of aggression.

I also found a significant interaction between time and treatment on the proportion of aggressive calls ($F_{1.6, 32.6} = 8.81$, $p = 0.001$; $\eta^2 = 0.31$; Table 1) with CORT-injected individuals exhibiting a greater increase in the proportion of aggressive calls produced over time relative to saline-injected males (Fig. 7). Furthermore, there was a significant main effect of social context on the proportion of aggressive calls ($F_{1, 20} = 16.95$, $p = 0.001$; $\eta^2 = 0.46$; Table 1) indicating that males have a greater propensity to call aggressively during STIs (Fig. 7). There was a marginal interactive effect of social context and treatment on the proportion of aggressive calls produced

($F_{1, 20} = 4.03$, $p = 0.06$; $\eta^2 = 0.17$; Table 1) with CORT-injected males exhibiting a greater increase in the proportion of aggressive calls relative to saline-injected males in the presence of a STI (Fig. 7).

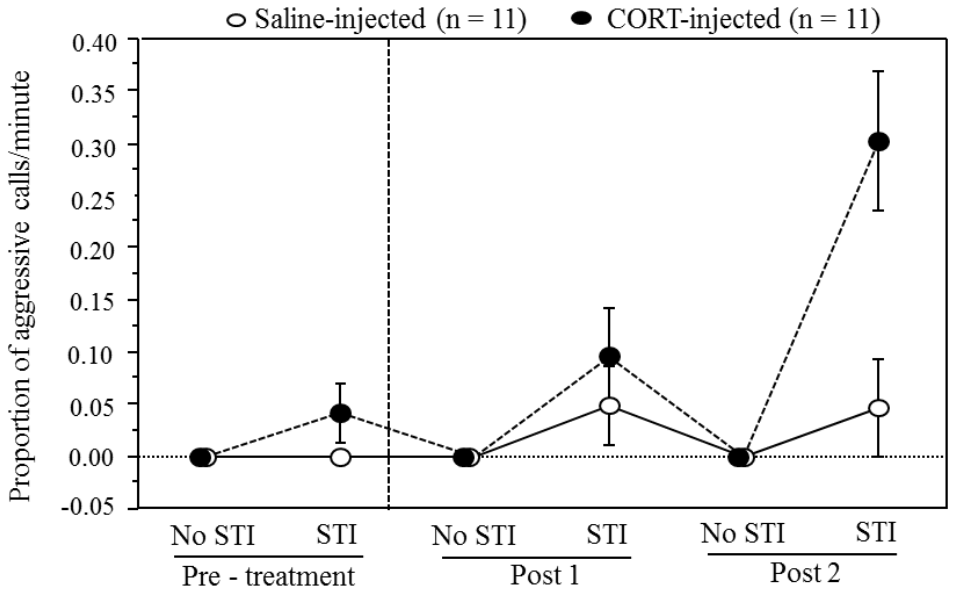

Figure 7. Line graph showing the proportion of aggressive calls prior to treatment (pre-treatment) and up to 65 min post-treatment and in the presence or absence of a simulated territorial intrusions (STI) for saline and CORT-injected males; the vertical dotted line represents approximate time of injection. The sampling period was designated in the same fashion as those for both temporal and spectral properties shown in figure 5 and 6. Whiskers indicate ±1 SE.

The effects of CORT on the probability of calling abandonment

First, I ran a Fisher's exact test using males sampled in the non-replicated design to determine whether there is an association between CORT administration and the probability of calling abandonment. None of the saline-injected males abandoned calling behavior while 11 of 12 (92%) of the CORT-injected males abandoned vocalization (Table 2). There was a statistically significant association between CORT administration and the probability of calling abandonment (sampling zeros were entered as 0.5 in the analysis, Higgins and Green, 2008;

Fisher's exact test; $p < 0.0001$). Next, I ran a second Fisher's exact test with the addition of social context (i.e., STI or no STI) using CORT-injected males from both the non-repeated measures design and the design with repeated measures. Males that were used in the repeated measures design were left isolated for the entirety of the experiment whereas males in the non-repeated measures design were subject to STIs. Of the CORT-injected males that were isolated, 4 of 15 (27%) abandoned vocalization (Table 2). There was a statistically significant association between social context and the probability that a CORT-injected male abandoned vocalization (Fisher's exact test; $p = 0.001$).

Table 2. The percentages of saline and CORT-injected males that abandoned calling behavior post-injection and in the presence or absence of a simulated territorial intrusion (STI).

Injection and social context	N	Stayed Caller	Abandoned Calling	% that abandoned calling
Saline-injected + STI	12	12	0	0%
CORT-injected + STI	12	1	11	92%
CORT-injected + No STI	15	11	4	27%

Discussion

Elevated CORT decreased attractiveness of male advertisement call

The results of this study indicate that acute elevations in circulating glucocorticoid (i.e., CORT) levels in male *H. cinerea*, simulating CORT production during male-male vocal interactions, rapidly mediate a suite of effects on courtship signals and behavior that reduce the

probability of attracting females. These results thus support the hypothesis that acoustic signals function, in part, as endocrine-based weapons. One major effect of elevated CORT level on vocalization involved a rapid reduction in call duration, which deviated from that of saline-injected controls within 15 min of CORT administration. Males injected with CORT and saline responded to the presence of a STI in a similar fashion (i.e., by increasing call duration) but CORT-injected males consistently produced shorter duration calls. This CORT-induced decrease in call duration can significantly compromise male attractiveness. For example, female *H. cinerea* preferred calls of longer duration in dual speaker playback experiments using differences in call duration (160 ms vs. 120 ms; Gerhardt, 1987) that approximated the mean call duration reported here for males with low versus high plasma CORT levels (i.e., pre-CORT injection mean call duration = 151 ms, post-CORT injection mean call duration = 133 ms).

In contrast to the effects of CORT injections on call duration, there was no evidence of a significant interaction between time and treatment on call rate. However, there was a context-dependent effect of CORT and saline injections on this vocal parameter; CORT-injected males increased their call rate in the absence of a STI and decreased their call rate the presence of a STI to a greater extent than saline-injected individuals. Such CORT-induced changes in call rate tracked changes in call duration, so that an increase in call rate was accompanied by a decrease in call duration. This effect is important because female preferences for high call rate (Gerhardt, 1991; Gerhardt and Huber, 2002; Davis and Leary, 2015) could outweigh the negative effects of elevated CORT level on call duration, resulting in a negligible impact on the attractiveness of males with high versus low CORT levels. However, CORT administration resulted in an overall reduction in vocal effort, indicating that CORT-mediated effects on call rate did not translate into increased energy invested in vocalization. This occurred because the CORT-mediated increase in

call rate was a consequence of shorter duration calls, not shorter intervals between successive calls. In other words, CORT administration caused a decrease in call duration but intercall duration remained largely unaffected by treatment, resulting in a net increase in call rate but decrease in vocal effort. CORT effects on intercall duration were only observed just prior to vocal cessation when the intercall duration increased relative to saline-injected controls, resulting in a more profound decrease in vocal effort in males with high CORT.

The overall reduction in vocal effort is critical because females of most anurans and other vertebrate species characteristically show preferences for more energetically demanding signals (Andersson, 1994; Gerhardt and Huber, 2002). The potential costs for males with high CORT levels were compounded by context-dependent effects. For example, the CORT-mediated increase in call rate in the absence of a STI disappeared in the presence of a STI, suggesting even greater costs for males with high CORT levels when interacting with other courting males, which occurs frequently in natural choruses (Leary et al., 2015).

In contrast to temporal call characteristics that are subject to directional selection via mate choice by females, there were no clear CORT-mediated effects on spectral call features that are under stabilizing selection via female preferences.

Elevated CORT increases aggression

The results also demonstrated that elevations in circulating CORT levels increase both the propensity for males to call aggressively and the proportion of aggressive calls produced. Female *H. cinerea* show a preference for advertisement over aggressive acoustic signals (*reviewed* by Gerhardt and Huber, 2002). For example, the addition of aggressive pulses at the onset of an advertisement call can decrease the attractiveness of the advertisement call in the

presence of females (Gerhardt, 1978a). Thus, male signalers presumably benefit by stimulating CORT production in rival males because CORT-mediated stimulation of aggression can reduce the attractiveness of vocal competitors, as shown in the present study. However, signalers would concurrently decrease their own attractiveness by calling aggressively during their attempt to decrease rival male investment in reproduction. In natural choruses, males may avoid this conflict with the addition of a pulsed prefix at the onset of advertisement calls (Leary, 2014). The pulsed prefix is generally different in duration and rate of repetition from standard aggressive calls (Oldham and Gerhardt, 1975). Moreover, the presence of the prefix does not appear to decrease the attractiveness of the advertisement call (Gerhardt, 1978b) but may stimulate CORT production among male competitors (Leary, 2014). Currently, however, the potential function of the pulsed prefix in the context of male-male interactions is unknown.

Elevated CORT increased the probability of satellite tactic expression

CORT injections increased the likelihood that a male abandoned calling behavior primarily during social interactions indicating that effects of CORT on tactic expression are context-dependent. If a highly stress responsive male exhibits increased CORT during a social challenge and the quality of his vocalizations decreases, then it may be in the best interest of the stressed male to abandon calling behavior and adopt an alternative mating tactic to increase mating opportunities. While calling males generally have a higher mating success relative to satellites, satellite males do occasionally acquire mates (Perrill et al., 1978; Miyamoto and Cane, 1980; Perrill et al., 1982; Arak, 1988; Perrill and Magier, 1988; Krupa, 1989; Haddad, 1991; Bourne, 1992; *reviewed* by McCauley et al., 2000). For instance, in *H. cinerea*, satellite males have a 43% mating success rate (Perrill et al., 1982). Conversely, if a male was to abandon

calling behavior in the absence of a nearby attractive conspecific, then the chance of acquiring a mate might be close to, if not, zero. This context-dependency of CORT on the abandonment of calling behavior may provide an explanation for why satellite males sometimes adopt vocal behavior after removal of the host (see Perrill et al., 1982; Arak, 1988; Humfeld, 2008).

The effects of CORT occurred independently of changes in circulating androgen levels

The results indicate that changes in signal quality and calling behavior in CORT-injected males occurred independently of changes in circulating levels of androgens. Mechanistically, elevations in CORT may override the effects of androgens by acting directly on the central nervous system to decrease the probability of calling behavior in anurans (see Leary, 2016). For instance, CORT potentially influences calling behavior by altering the secretion of arginine vasotocin (AVT) from motor neurons in the vocal motor pathway of the anuran brain (Leary et al., 2006a). Androgens are most notably associated with this system, as they appear to influence the quantity and distribution of AVT receptors in the brain (Rose and Moore, 2002). Furthermore, AVT administration often elicits calling behavior in anurans (Propper and Dixon, 1997; Chu et al., 1998) as well as amplectic clasping in rough-skinned newt, *Taricha granulosa* (Moore and Miller, 1984). Conversely, elevated glucocorticoids are known to suppress amplectic clasping in *T. granulosa* (Rose and Moore, 2002; Moore and Miller, 1984; Davis et al., 2015). Therefore, it seems possible that CORT may influence calling behavior in male anurans by inhibiting the release of AVT from vocal motor neurons, or by altering androgen-receptor levels in the brain, indirectly influencing the release of AVT from motor neurons.

Conclusion

The acute stress response plays a central role in mediating tradeoffs between self-maintenance behavior and reproduction (Sinervo and Svensson, 1998; Wingfield et al., 1998; Cote et al., 2006; Breuner et al., 2008; Bonier et al., 2009; Hau eta al., 2010; Crespi et al., 2013; Patterson et al., 2014). Currently, however, we have a poor understanding of how these trade-offs unfold in the context of intra- and intersexual selection. Here, I build upon the "acute-stress framework" in the context of sexual selection using green treefrogs, *H. cinerea*. I predicted that acoustic-based stimulation of CORT production among competing males functions in an armament capacity by compromising rival male energetic investment in calling behavior and, hence, vocal attractiveness. Consistent with this prediction, experimentally elevated CORT decreased vocal effort and increased aggressive signaling and the probability of vocal abandonment—all of which potentially confer fitness disadvantages by decreasing the chances of attracting females. These CORT-mediated effects occurred independently of any detectable changes in circulating androgen levels and were most prominent when males were challenged by a STI, indicating that stress-mediated effects are context dependent. Together, these results show that stress hormones can modulate the expression of male sexual signals, and are a target of both inter- and intrasexual selection.

CHAPTER TWO

BIDIRECTIONALITY OF HORMONE-BEHAVIOR RELATIONSHIPS AND SATELLITE CALLER DYNAMICS IN MALE GREEN TREEFROGS: MULTIPLE FACTORS MEDIATE MATING TACTIC EXPRESSION

Abstract: Whether hormonal differences among males that conditionally alternate between mating tactics are a cause or consequence of behavioral expression is central to understanding the mechanisms regulating the adoption of a particular tactic. This issue is rooted in the bidirectionality of hormone-behavior relationships and is pertinent to alternative mating tactics in anurans because the social-acoustic environment can mediate changes in both tactic expression and hormone levels. Hence, it is not clear whether males adopt different tactics in response to rival male signals, hormone levels, or both. Here, I address this problem in male green treefrogs, *Hyla cinerea*, using vocal playback experiments combined with measures of circulating hormone levels, body size, condition, and plasma glucose levels. Despite evidence that hormonal differences between calling and satellite males can arise during social interactions and are casually associated with tactic expression in this species, results revealed that hormone level did not predict mating tactic expression in vocal playback experiments. For example, 14 out of 68 calling males in natural choruses (21%) adopted non-calling satellite behavior in response to broadcast advertisement calls; these males produced less attractive calls, were smaller, and in poorer body condition than males that continued to call, but did not differ in corticosterone, androgen, or plasma glucose levels. Results thus suggest that vocal quality, body size and/or

condition play a central role in tactic expression and that differences in circulating hormone levels for callers and satellites in natural choruses potentially arise as a consequence of mating tactic expression. Together, the results suggest that there may be no clear dichotomy in the "cause versus consequence" debate revolving around hormonal mediation of alternative mating tactics because both hormone levels and the social environment can contribute to individual variation in mating tactic expression.

Introduction

Competition among males can promote the evolution of intraspecific variation in male mating behavior, collectively referred to as alternative mating tactics (AMTs; Dominey, 1984; Andersson, 1994; Gross, 1996; Brockmann, 2001; Taborsky et al, 2008; Shuster, 2010). AMTs generally involve distinct differences among males in the energy allocated to courtship behavior — males adopting the alternative tactic typically invest less energy in courtship and achieve reproductive success by exploiting the energetic investment of males employing the "dominant" tactic (Taborsky et al., 2008). Such variation in male mating behaviors is phylogenetically widespread and diverse (Gross and Charnov, 1980; Waltz, 1982; Eberhard and Gutierrez, 1991; Thompson et al., 1992; Lank et al., 1995; Sinervo and Lively, 1996) and may arise as a consequence of fixed genetic polymorphisms or may represent conditional tactics within genetically monomorphic individuals (*reviewed* by Gross 1996; Oliveira et al., 2008). For example, fixed variation in mating behavior in male marine isopods (*Paracerceis sculpta*) is attributable to a polymorphism at a single autosomal locus that is maintained by equal fitness payoffs (Shuster and Wade, 1991). Conversely, conditional tactic switching potentially optimizes reproductive success for males that are unable to effectively compete with conspecific males

employing the “dominant” tactic (Dominey, 1984; Andersson, 1994; Gross, 1996; Brockmann, 2001; Taborsky et al., 2008; Shuster, 2010). Males that adopt alternative conditional tactics are often less successful at acquiring mates than males that employ the dominant tactic, but adoption of the alternative tactic is expected to result in the greatest probability of reproductive success for competitively inferior males (Gross, 1996).

Mating tactic switch points

Mechanisms governing the adoption of condition-dependent mating tactics often involve dynamic interplay between an individual’s genes, environment, and physiological state (Andersson, 1994; Gross, 1996; Shuster and Wade, 2003; Oliveira et al., 2008). In this respect, considerable emphasis has been placed on genetic variation in "tactic switch-points". For instance, the environmental threshold model (Hazel et al., 1990; Gross, 1996; Tomkins et al., 2004; Tomkins and Hazel, 2007; Buzatto et al., 2012) proposes a norm of reaction in which tactic expression is influenced by an environmental cue whose value determines the threshold at which a particular phenotype is produced (Tomkins and Hazel, 2007). While there is evidence of genetic variation in switch-point sensitivity among males that conditionally alternate between mating tactics (Radwan, 1995; Tomkins et al., 2004; Piché et al., 2008; Buzatto et al., 2012), the physiological mechanisms regulating these switch-points are often poorly understood (Oliveira et al., 2008). Such information is, however, central to understanding how alternative mating behaviors are maintained in natural populations (Brockmann, 2001).

Hormones are often implicated as key factors governing mating tactic switch-points (Moore, 1991; Oliveira et al., 2008; Lema and Kitano, 2013). For instance, hormones can influence tactic expression by organizing target tissues during critical stages of development that

permanently affect the phenotype (i.e., "organizational effects" of hormones, Moore, 1991; Oliveira et al., 2008). Male tree lizards (*Urosaurus ornatus*) administered androgens on the day of hatching, for example, develop into a fixed territorial orange-blue morph, whereas males castrated on the day of hatching develop into a fixed non-territorial orange morph (Hews et al., 1994). Alternatively, hormones can have "activational" effects on the phenotype that mediate transitions between conditional "plastic" tactics throughout the life of the organism (Moore, 1991; Oliveira et al., 2008). Within the orange morph of *U. ornatus*, for example, tactic switching between sedentary and nomadic behavior is mediated by changes in circulating corticosterone (CORT) level (Moore et al., 1998). Importantly, early organizational effects of hormones can give rise to individual variation in responsiveness/sensitivity to activational effects of hormones later in life (Phoenix et al., 1959; Arnold and Breedlove, 1985; Arnold, 2009). Such organizational and activational effects of hormones form the crux of the Relative Plasticity Hypothesis (RHP; Moore, 1991; Thompson and Moore, 1992; Moore et al., 1998) which has been instrumental in directing research aimed at understanding the mechanistic basis for AMTs.

The RPH predicts that activational effects of hormones mediate the expression of plastic adult reproductive phenotypes (Moore, 1991). While there is considerable evidence indicating hormonal differences among males employing different conditional/plastic mating tactics (Silverin and Wingfield, 1982; Thompson and Moore, 1992; Brantley et al., 1993; Clarke and Faulkes, 1998; Uglem et al., 2003; Leary et al., 2004; Wikelski et al., 2005; Spinney et al., 2006; Schradin et al., 2009; Leary and Harris, 2013), such differences alone do not demonstrate that hormones are causally associated with mating tactic expression (Oliveira et al., 2008). For example, males employing different conditional tactics may possess disparate hormone levels if the environmental/social conditions associated with the alternative tactic result in changes in

hormone levels (Fig. 1). This issue is rooted in the bidirectionality of hormone-behavior relationships: hormones can alter the probability that a behavior is expressed, but the expression of certain behaviors creates new social dynamics that can, in turn, influence circulating hormone levels (Oliveira et al., 2002; Oliveira, 2004; 2005 Adkins-Regan, 2005). Hence, it is often unclear whether differences in circulating hormone levels among males practicing conditional tactics are a cause or consequence of tactic expression (Oliveira et al., 2008; Fig.1).

Here, I address the problem of bidirectionality of hormone-behavior relationships in the context of AMTs in male green treefrogs, *Hyla cinerea*. As with males of many anuran species, male *H. cinerea* conditionally alternate between a dominant calling tactic and an alternative non-calling "satellite" mating tactic (Perrill et al., 1978; Perrill et al., 1982; Humfeld, 2008; see Chapters 1 and 3). Satellite males characteristically remain near calling "host" males and attempt to intercept females attracted to the host's calls (Sullivan, 1982; Fig. 2). Although AMTs are taxonomically widespread in anurans (e.g., bull frog, *Rana catesbiana*, Howard, 1978; grey treefrog, *Hyla versicolor*, Fellers, 1979; hourglass treefrog, *Hyla ebraccata*, Miyamoto and Cane, 1980; cricket frog, *Pseudacris crucifer*, Forester and Lykens, 1986; Great Plains toad, *Bufo cognatus*, Leary et al., 2004), the mechanisms mediating transitions in male mating behaviors are poorly understood (*reviewed* by Leary, 2009).

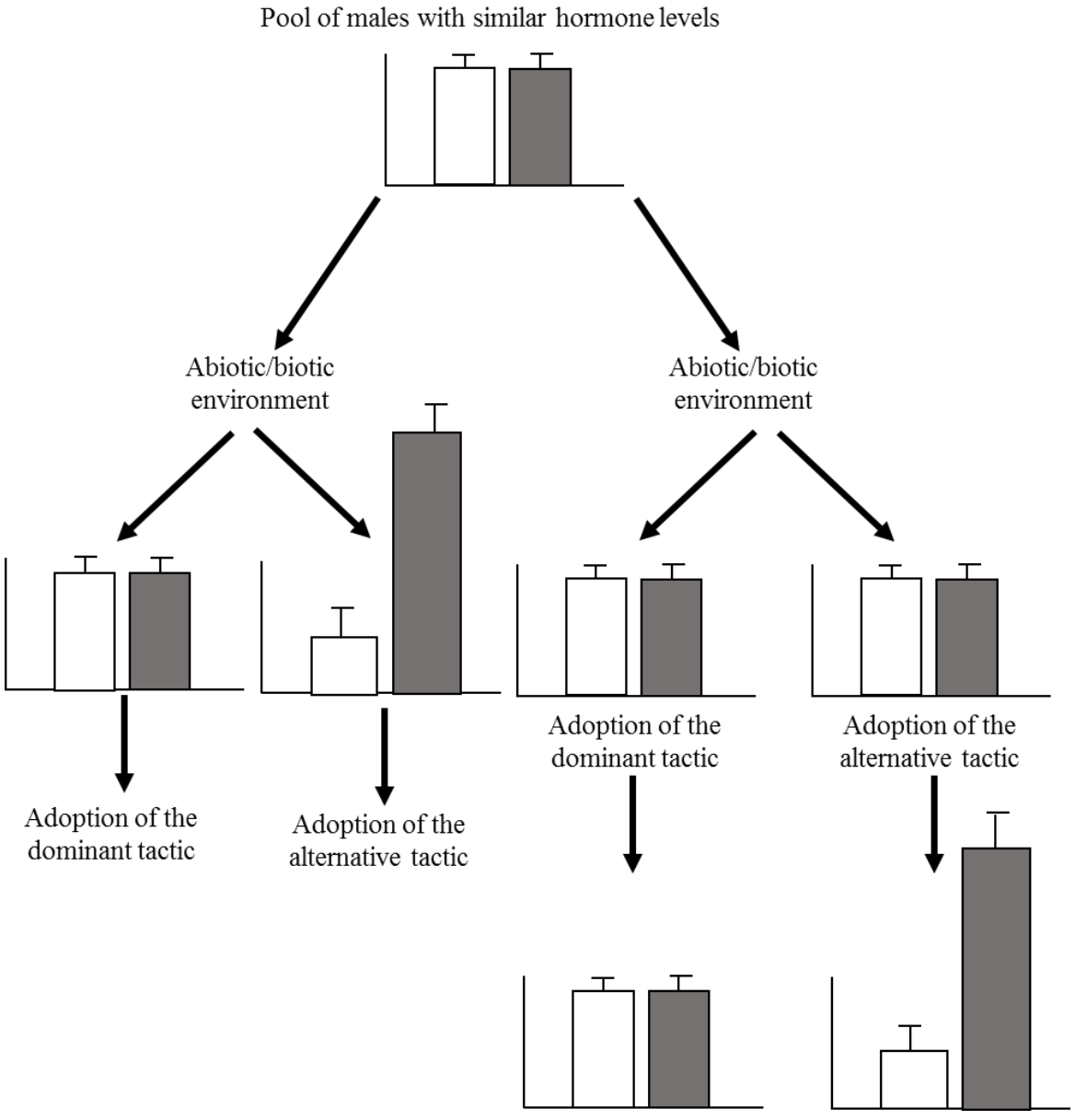

Figure 1. The importance of potential bidirectionality of hormone-behavior relationships in the context of alternative mating tactics. (A) Differences in hormone levels are causally associated with mating tactic expression. (B) Differences in behavioral phenotypes are a consequence of mating tactic expression. Bars are hypothetical circulating levels of sex (white bar) and stress steroids (grey bar).

Figure 2. A natural satellite (left) - caller (right) association in *H. cinerea*.

There are two primary hypotheses to explain mating tactic expression in anurans that make this group ideally-suited for probing the social and hormonal basis for mating tactic expression. One prevailing hypothesis (referred to here as the "relative attractiveness hypothesis") proposes that mating tactic expression is governed by the relative attractiveness of neighboring males (Waltz, 1982; Arak, 1988). This hypothesis predicts that males adopt a satellite tactic when the attractiveness of a nearby calling male's vocalizations sufficiently exceeds their own attractiveness. Unattractive males may thus increase their chances of acquiring mates (and simultaneously conserve energy) by "parasitizing" more attractive males displaying the dominant tactic (Perrill et al., 1978; 1982; Waltz, 1982; Arak, 1988; Perrill and Magier, 1988; Halliday and Tejedo, 1995; Wells, 2001; Leary et al., 2004; Leary et al., 2006a; 2006b; Humfeld, 2008; Castellano et al., 2009; Berec and Bajgar, 2011). The second hypothesis is

rooted in the energetic demands of vocalization and hormonal regulation of calling behavior. For example, the Energetics–Hormone Vocalization (EHV) model predicts that energy expended while vocalizing (Pough et al., 1992; Prestwich, 1994) drives elevations in circulating levels of the stress hormone corticosterone (CORT; i.e., the primary glucocorticoid in amphibians) that negatively affects the production of androgens and decreases the probability of calling behavior (Emerson, 2001). The “relative attractiveness hypothesis” thus predicts that satellite and calling males differ in vocal attractiveness but do not necessarily differ in circulating hormone levels. In contrast, the EHV model predicts that non-calling satellite males are in poorer condition (i.e., are depleted of available energy reserves), have higher CORT levels, lower androgen levels, and are less attractive than callers.

Identifying the factors that mediate changes in tactic expression

The conventional approach to testing the "relative attractiveness hypothesis" involves the use of vocal playback experiments wherein the behavior of calling male anurans is observed during exposure to broadcast advertisement calls that simulate an approaching male (Perrill et al., 1982; Robertson, 1986; Arak, 1988; Perrill and Magier, 1988; Wagner, 1992; Kadadevaru and Kanamadi, 2002; Park and Cheong, 2002). Males that adopt non-calling satellite behavior in response to the broadcast stimulus are thought to represent a class of competitively inferior/less attractive males compared to males that continue to call in response to the playback stimulus (Perrill et al., 1982; Arak, 1988; Perrill and Magier, 1988).Yet, there is little conclusive evidence supporting this hypothesis because no such studies have actually examined potential differences in vocal attributes of males that continue to call in response to a broadcast stimulus versus those that do not. However, there is indirect support for this hypothesis from experiments that

manipulate satellite-caller associations. For example, satellite male *H. cinerea* that were induced to call by removal of associated calling host males produced calls that were shorter in duration and possessed lower carrier frequencies than calling host males (Humfeld, 2008). Here, the issue of bidirectionality of hormone-behavior relationships is again critical in interpreting whether such differences in vocalization are causally or consequentially related to mating tactic expression. For example, the social-acoustic environment experienced by satellite males could influence circulating hormones levels that, in turn, alter vocalization. For instance, males of this species produce aggressive vocalizations that cause elevations in circulating glucocorticoids and a reduction in androgens (Leary, 2014) that mirror the hormonal profiles of callers and satellites in natural choruses (Leary and Harris, 2013). Elevated glucocorticoid levels (characteristic of satellite males), in turn, cause a reduction in the duration of male advertisement calls and can also contribute to a reduction in the carrier frequency of the calls (see Chapter 1), paralleling reported differences in vocal attributes for callers and satellites. Moreover, elevated glucocorticoid levels cause a reduction in the probability of calling behavior and the adoption of satellite behavior (see Chapter 1). Hence, it is not clear whether male mating tactic expression is related to differences in hormone levels, vocal attractiveness or both.

To address this problem, I combined vocal playback experiments (using *H. cinerea*) with measures of circulating levels of CORT, androgens and vocal parameters (i.e., call duration, calling rate, vocal effort, peak carrier frequencies; see Chapter 1 for review of these parameters) known to be important in mate choice by females of this species (Gerhardt, 1974; 1978a; 1982; 1987; 1991; Gerhardt et al., 1990; Gerhardt and Huber, 2002; Humfeld, 2008). Because satellite male *H. cinerea* possess higher CORT and lower androgen levels compared to calling males (Leary and Harris, 2013) and elevated CORT level increases the propensity for non-calling

behavior (see Chapter 1), I predicted that males adopting satellite behavior in response to broadcast advertisement calls would have higher CORT and lower androgens than males that continue to call in response to the broadcast advertisement call. Furthermore, I predicted that these hormonal differences would result in decreased vocal attractiveness in males that adopt satellite behavior in response to broadcast advertisement calls. Such outcomes would provide support for a causal association between hormone level and mating tactic expression. In contrast, the lack of significant differences in circulating hormone levels for males that continue and stop calling in response to broadcast advertisement calls would suggest that mating tactic expression is potentially determined by differences in vocal attractiveness and that differences in circulating hormone levels of satellites and callers in natural choruses (Leary and Harris, 2013) is a consequence of mating tactic expression.

I also included measures of body size, body condition and plasma glucose levels because these variables have either been implicated as primary determinants of mating tactic expression or are correlated in some way with vocal attributes and/or circulating hormone levels. For instance, satellite males are often smaller and in poorer body condition than associated calling males (Forester and Lykens, 1986; Eggert and Guyetant, 2003; Leary et al., 2005; Humfeld, 2008; Castellano et al., 2009; Berec and Bajgar, 2011; Brepson et al., 2012), and both body size and condition can influence circulating stress hormone levels (Dunlap and Wingfield, 1995; Kitaysky et al., 1999; Jessop et al., 2002; Leary, 2014; *reviewed* by Moore and Jessop, 2003; Husak and Moore, 2008) and vocal parameters (Leary, 2006b; see Chapter 1). Thus, I predicted that in addition to hormonal differences, males that adopt a satellite tactic in response to broadcast advertisement calls would also be smaller and in poorer body condition than males that continued to call. Lastly, I predicted that males adopting satellite behavior would also have

higher plasma glucose levels because of the gluconeogenic effects of high glucocorticoids on hepatic metabolism which increases glucose production (see Exton, 1979).

Methods

General experimental design and vocal playback experiments

Calling *H. cinerea* males were studied in natural choruses at the University of Mississippi Field Station (Lafayette County, MS, USA) throughout peak hours of chorus activity (~2000-2200 hour) during the 2014 and 2015 breeding seasons. Calling males were located (using low-powered headlamps to minimize disturbance) and individual body temperatures were measured to the nearest 0.1ºC (range 22.0 – 26.0ºC). A series of 30 consecutive calls were then recorded from individual males using a Marantz PMD 222 cassette recorder equipped with a Sennheiser directional condenser microphone (Model ME-66). After vocal recordings, calling males were immediately exposed to a white noise control stimulus and/or a broadcast conspecific advertisement call and behavioral responses (i.e., whether males continued to call or adopted non-calling satellite behavior) were assessed and recorded. The broadcast advertisement call stimulus consisted of a single isolated call possessing the mean call parameters for this population (e.g., dominant carrier frequencies = 800 and 2600 Hz, call duration = 189 ms, intercall duration = 612 ms; Fig. 3A; Davis and Leary, 2015). Advertisement calls were repeatedly broadcast for a 5-min focal period from a distance of approximately 30 cm using a hand-held speaker (see Arak, 1988). The control stimulus consisted of white noise with an amplitude envelope similar to that of the advertisement call stimulus and consisted of the same duration and inter-stimulus duration as the advertisement call stimulus (Fig. 3B). The purpose of the white noise stimulus was to assess the specificity of male responses to conspecific calls. Both

stimuli were broadcast at an amplitude of approximately 90 dB (measured with a hand-held sound pressure level meter, Ivie Technologies Inc., model IE-45, fast weighting setting), approximating the amplitude of natural calls for this species (Humfeld, 2013). The standardized advertisement call stimulus was broadcast to 68 calling male *H. cinerea* in natural choruses while the white noise control stimulus was presented to a subset of 20 calling males used in the experiment.

Acquisition of blood for hormone analysis

Immediately following playback experiments and assessment of behavioral responses, approximately 75–100μL of blood was obtained via cardiac puncture using a 28-gauge heparinized needle. Snout-ischial length (SIL) and weight were then measured and males were marked on the venter using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL, USA) for future identification (i.e., after release at the site of capture). Acquisition of blood was generally completed in 4 min or less, while body size and weight measures and application of tattoos was completed within 10 min; none of these procedures resulted in immediate or long term overt effects on male reproductive behavior (see also Leary and Harris, 2013). Blood samples were stored on ice until returned to the lab (~2-4 h later), where they were centrifuged for 12 min at 2200 rpm to separate plasma. Plasma samples were transferred to a separate eppendorf tubes and stored in a -20ºC freezer until dihydrotestosterone (DHT), testosterone (T), and corticosterone (CORT), levels were quantified using column chromatography and radioimmunoassay (described below).

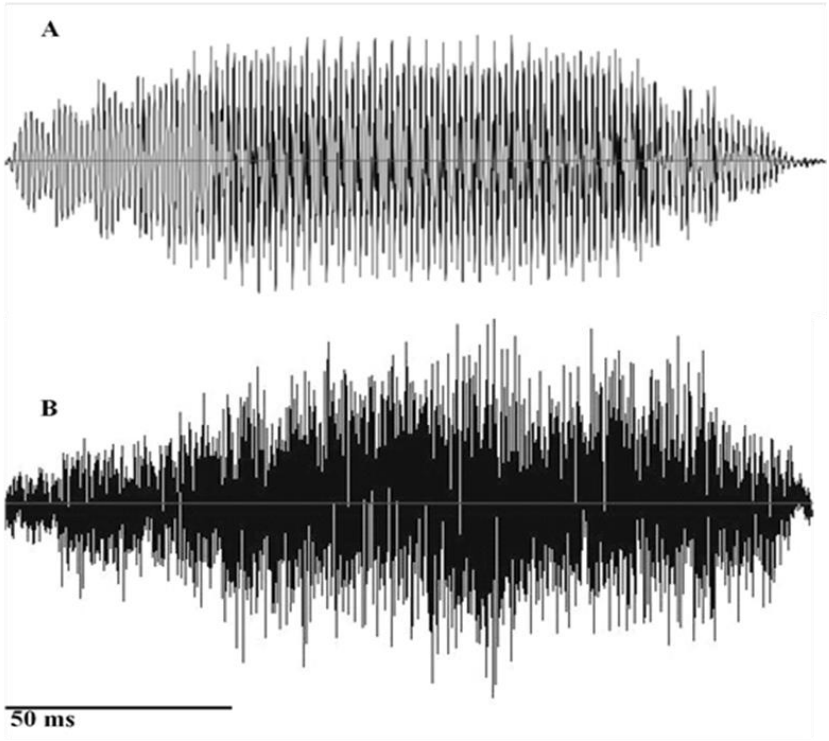

Figure 3. (A) Waveform representation of the standard advertisement call and (B) the control white noise stimulus used in the vocal playback experiment.

Vocal analysis

Calls were analyzed using Raven Pro acoustics software (Cornell Bioacoustics Laboratory). Acoustic parameters known to be important in mate choice by females Gerhardt, 1974; 1978b; 1982; 1987; 1991; Gerhardt et al., 1990; Gerhardt and Huber, 2002; Humfeld, 2008) were measured from the middle portion of 5-10 consecutive calls; these included measurements of call duration and intercall duration (ms), call rate (calls/min), as well as low and high dominant carrier frequencies (kHz). I then calculated vocal effort (i.e., proportion of time spent calling) by dividing call duration by the sum of call duration and intercall duration. Vocal effort was used as a proxy for energy expended during vocalization because the proportion of time spent vocalizing contributes to overall acoustic energy and is positively correlated with

metabolic rate (see Taigen and Wells, 1985; Pough et al., 1992; Prestwich et al., 1994). Amplitude (dB) was not included in this analysis since accurate sound intensity was difficult to obtain in many cases (i.e., males often call from dense thickets of vegetation that prevented consistent orientation of the SPL meter), and satellites produce calls with similar intensity compared to other calling males (Leary et al., 2006a; Humfeld, 2008).

Body condition calculations and blood glucose estimates

Body condition estimates and plasma glucose concentrations were used as proxies for energy reserves. Body condition was calculated using the residual values from a linear regression of the cubed root body weight on SIL divided by SIL (see also Baker, 1992; Leary et al., 2008). Plasma glucose levels were measured using a standard glucose meter (Relion Prime Glucose Monitoring System, USA). Approximately 0.05µl of each sample was tested twice for blood glucose level and the two values did not differ (paired samples *t*-test: $t_{(53)} = -0.06$, $p = 0.95$). Hence, the mean blood glucose value was used in statistical analysis.

Sample preparation, column chromatography, and radioimmunoassay procedures

Hormone separation and quantification of hormone concentrations followed the methods described in Leary et al. (2004). Briefly, 30µl of each individual plasma sample was allocated into separate extraction tubes and incubated overnight with radiolabeled hormone (PerkinElmer, Inc. Hebron, Kentucky). Steroids were then extracted from the plasma using diethyl ether and subsequently dried under nitrogen gas at 40ºC. Dried samples were then resuspended in 10% ethyl acetate in iso-octane and loaded onto diatomaceous earth columns containing a 3:1 diatomaceous earth:distilled water "glycol trap" and a 1:1 propanediol:ethylene glycol mixture.

Mixtures of 10%, 20%, and 52% ethyl acetate in iso-octane were then used to collect DHT, T, and CORT, respectively. Fractions were dried under nitrogen gas and resuspended in phosphate buffered saline containing 0.3% gelatin for use in radioimmunoassay. T antibody was purchased from Fitzgerald Industries International, Inc. (Acton, Massachusetts) and used to measure both DHT and T. The CORT antibody was purchased from MP Biomedicals, LLC (Solon, Ohio). All samples were assayed in duplicate.

Plasma samples were analyzed for DHT, T and CORT levels in 2 assays. Mean intra-assay coefficients of variation for DHT, T, and CORT were 4.6%, 2.3%, and 9.5%, respectively, based on 4 standards run with each assay. Inter-assay coefficients of variation for DHT, T, and CORT were 6.8%, 14%, and 11%, respectively.

Statistical analysis

Prior to conducting parametric analyses, I tested all variables for homogeneity of variance and normality. Body temperatures can alter various attributes of male advertisement calls in anurans (Gerhardt and Huber, 2002); thus, a simple linear regression analysis was performed on all measured acoustic parameters against body temperature. Vocal parameters that were significantly correlated with temperature (i.e., call duration and intercall duration) were corrected to the mean recording temperature (24ºC) by calculating the residual values obtained from the linear regression and using the linear equations from the regression lines to calculate the adjusted value (Leary et al., 2008).

Simple linear regressions were performed with circulating levels of DHT, T and CORT against blood acquisition time to determine if blood acquisition time was related to circulating hormone levels. Next, I used a one-way analysis of variance (ANOVA) to compare circulating

levels of CORT and androgens, acoustic parameters, weight, body condition, and blood glucose for males that continued to call and males that adopted the satellite tactic in response to broadcast stimuli. Pearson correlation analyses were then performed to estimate relationships among the dependent variables. There were several cases with missing data and, hence, the correlation matrix was run using pairwise deletion in which missing values are removed on a "by-analysis" basis. This approach is useful for small sample sizes and increases the power of the analysis (Peugh and Enders, 2004; Graham, 2009). Note that Bonferroni adjustments were not made for the above analyses stemming from the likelihood of type II errors increasing and the possibility of deeming important biological differences non-significant (Perneger, 1998).

Lastly, a logistic regression analysis was performed to determine which of the predictor variables best predicted the behavioral response of males to broadcast stimuli. Variable selection for the regression analysis was determined using backward regression with a log likelihood ratio (Bursac et al., 2008). The full model began as an eleven-predictor model (i.e., SIL, condition, DHT, T, CORT, low and high carrier frequencies, call and intercall duration, call rate, and vocal effort) in which backward regression was used to determine the most significant covariates. The second-order Akaike information criterion (AIC_c) for small sample sizes was then calculated for each model using the equation (see Sugiura, 1978; Hurvich and Tsai, 1989):

$$AICc = -2LL + 2K + \frac{2K(K+1)}{(n-k-1)}$$

Here, -2LL is the log likelihood ratio, *K* is the model degrees of freedom for the predictor variables (*p*) + 1 and *n* is the sample size. The AIC function penalizes for the addition of parameters and thus helps to select the most parsimonious model; the model with the lowest

AIC_c score is considered the best model among all models specified (Kullback and Leibler, 1951). The reduced model with the lowest AIC score was chosen as the best fit model for the analysis which included the predictor variables SIL, condition, CORT, high carrier frequency, intercall duration, call rate and vocal effort. Multicollinearity was then tested by estimating variance inflation factors (VIF) which assess the degree of correlation among variables (O'Brien, 2007); a VIF > 10 is indicative of robust multicollinearity (Mason et al., 1989). However, VIFs remained below 10 and thus none of the variables were removed from the reduced model.

A Hosmer and Lemeshow goodness-of-fit test was used to assess the fit of the model and a Nagelkerke R^2 was computed to assess the variance explained by the model (Meyers et al., 2006). The null hypothesis was tested using the Wald test which determines whether the regression coefficient of a predictor variable is significantly different from zero. The equation for the Wald statistic is below:

$$\text{Wald} = \frac{B}{SE}$$

Where B is the regression coefficient and SE is the standard error of the coefficient (Zeilhoffer et al., 2007). All statistical analyses were performed using Statview (SAS Institute, Inc.) and SPSS (IBM Software).

Results

A total of 54/68 males (79%) continued to call while 14/68 males (21%) stopped calling and adopted the satellite mating tactic in response to broadcast advertisement calls. The latter group was characterized by a crouched position typical of satellite males (see Sullivan, 1982; Perrill et al., 1982) and orientation towards the speaker. All subjects that were exposed to the white noise control stimulus (n = 20) continued to produce advertisement calls. However, when

exposed to the broadcast advertisement call, 9/20 (45%) of these males subsequently became aggressive and 3/20 (15%) adopted satellite behavior. These results suggest that males are less responsive to stimuli lacking the spectral structure of male advertisement calls (i.e., males are unlikely to be responding to arbitrary sounds).

Circulating hormone levels

Blood acquisition time was negatively correlated with circulating T levels ($r^2_{(65)} = 0.06$ $p = 0.05$; Fig. 4A) and positively correlated with CORT levels ($r^2_{(65)} = 0.18$, $p < 0.0001$; Fig 4B). Subsequent to removal of four males that took longer than 4 min to obtain blood, there was no longer a detectable decrease in T ($r^2_{(61)} = 0.04$ $p = 0.08$; Fig. 4A) or increase in circulating levels of CORT ($r^2_{(61)} = 0.00$ $p = 0.87$; Fig. 4B). Additionally, one male was not included in the analysis ecause a sufficient blood sample was not obtained for radioimmunoassay. Thus, this hormonal data, obtained from 63 of the 68 calling males, was used in hormonal analyses (see below).

Males that adopted the satellite tactic and males that continued to call in response to the advertisement call stimulus did not differ with respect to circulating levels of DHT ($F_{1,61} = 1.15$, $p = 0.29$, $\eta^2 = 0.02$), T ($F_{1,61} = 0.94$, $p = 0.34$, $\eta^2 = 0.02$), or CORT ($F_{1,61} = 2.43$, $p = 0.13$, $\eta^2 = 0.04$) indicating that variation in circulating hormone levels did not predict the response of males to broadcast advertisement calls (Table 1; Fig. 5). A post-hoc power analysis was conducted using the software package G*Power (Faul and Erdfelder, 1992) to estimate the sample size needed to detect a significant difference in circulating CORT levels between the two groups of males with a small effect size (Cohen's $f = 0.04$), an alpha level of 0.05 and a hypothetical power of 0.80. The post hoc power analysis indicated a total sample size of 4,908 individuals (~ 2454

per group) to detect a significant difference in CORT levels between the two groups of males (Fig. 6). For reference, choruses that were studied typically consisted of 30 males or less.

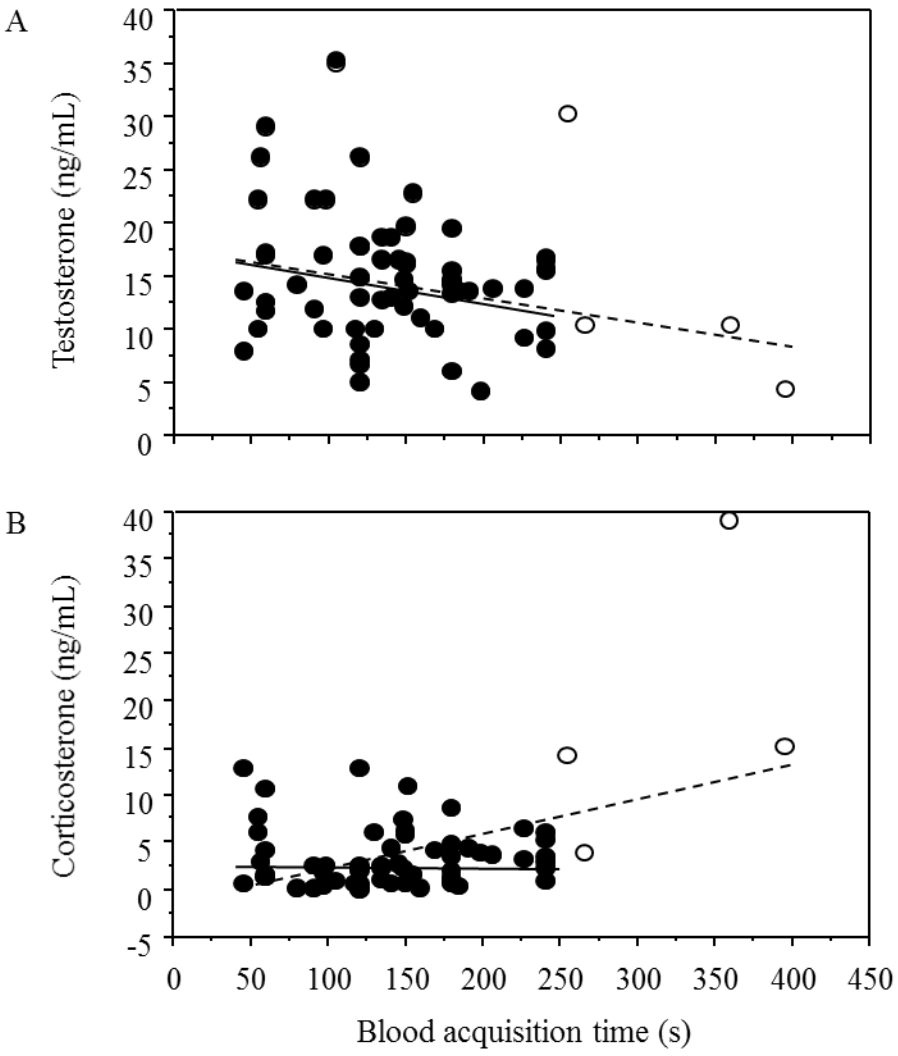

Figure 4. The relationship between concentrations of (A) testosterone and (B) corticosterone at the time of blood acquisition. The stippled line is the regression trend in which the hormone levels of all males are included. The solid line is the regression trend after the removal of the four males that took longer than 240 s (e.g., 4-min) to acquire blood (represented by open circles); these males were not used in statistical analyses involving hormone levels.

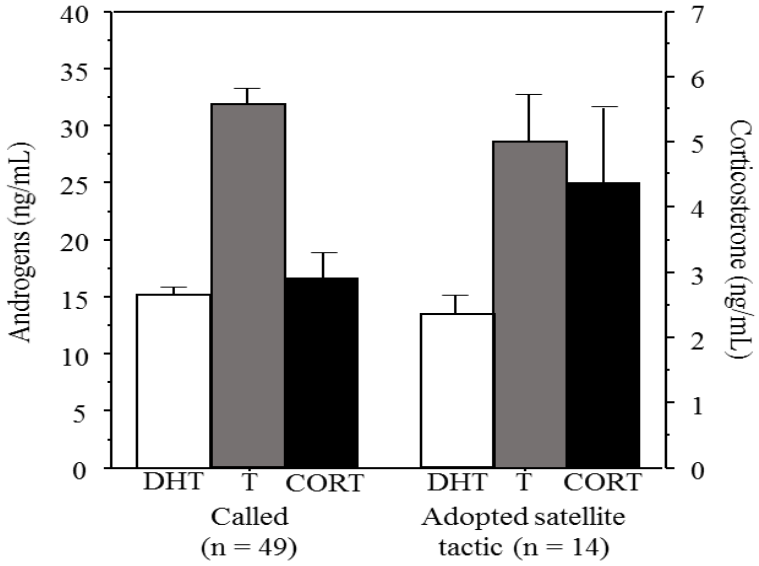

Figure 5. Circulating levels of dihydrotestosterone (DHT), testosterone (T), and corticosterone (CORT) in males that called in response to broadcast advertisement calls and males that adopted the satellite tactic. Whiskers indicate ±1 SE

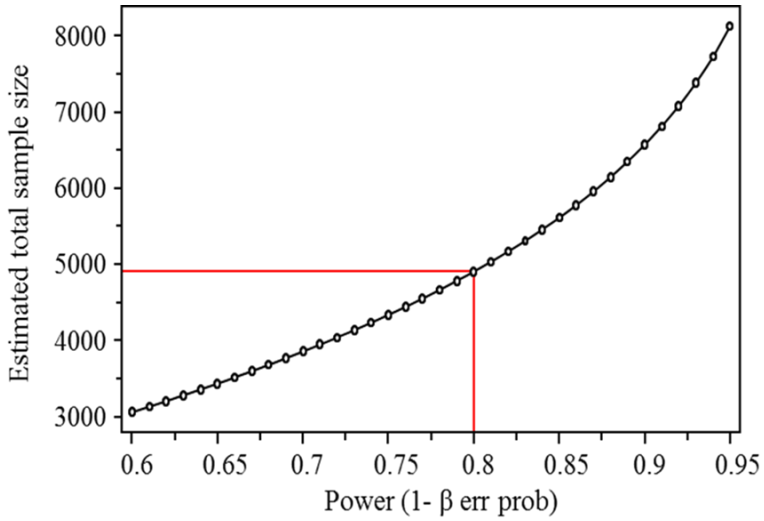

Figure 6. Sample size estimation from a post-hoc power analysis used to determine how large the total sample size would need to be to detect a difference in circulating levels of CORT between the two groups of males.

Advertisement call characteristics

Males that adopted the satellite tactic produced calls that were shorter in duration than males that continued to call in response to the broadcast advertisement call ($F_{1, 65} = 6.92$, $p = 0.01$; $\eta^2 = 0.10$; Table 1; Fig.7A) but did not differ with respect to intercall duration ($F_{1, 65} = 0.07$, $p = 0.80$; $\eta^2 = 0.001$; Table 1; Fig. 7A) or call rate ($F_{1, 65} = 0.06$, $p = 0.82$; $\eta^2 = 0.001$; Table 1; Fig. 7B). The net effect was that vocal effort was significantly lower in males that stopped calling compared to males that continued to call in response to broadcast advertisement call ($F_{1, 65} = 6.38$, $p = 0.01$; $\eta^2 = 0.089$; Table 1; Fig. 7B). Moreover, males that adopted the satellite tactic in response to the broadcast advertisement call produced calls (prior to broadcasting stimuli) with a higher low-frequency peak than males that continued to call ($F_{1, 65} = 4.06$, $p = 0.05$, $\eta^2 = 0.06$; Table 1; Fig. 7C). Conversely, the high-frequency peak of advertisement calls was not significantly different for males that stopped and continued to call in response to the broadcast advertisement call ($F_{1, 65} = 0.20$, $p = 0.65$, $\eta^2 = 0.003$; Table 1; Fig. 7C).

Aggression

Thirty-two of the 68 (47%) males exhibited aggressive behavior towards the speaker during exposure to the advertisement call stimulus. I performed a one-way ANOVA to compare circulating levels of CORT and androgens, as well as body size and condition between aggressive and non-aggressive males. Non-aggressive and aggressive males did not differ in circulating levels of DHT ($F_{1, 61} = 1.85$, $p = 0.18$; $\eta^2 = 0.03$), circulating levels of T ($F_{1, 61} = 1.85$, $p = 0.18$; $\eta^2 = 0.03$), or circulating levels of CORT ($F_{1, 61} = 0.02$, $p = 0.89$; $\eta^2 = 0.0003$). Furthermore, non-aggressive and aggressive males did not differ in SIL ($F_{1, 66} = 0.09$, $p = 0.77$; $\eta^2 = 0.001$) or condition ($F_{1, 66} = 0.23$, $p = 0.63$; $\eta^2 = 0.004$).

Body size and condition

Males that adopted the satellite tactic in response to the broadcast advertisement call were significantly smaller ($F_{1, 66} = 13.09$, $p = 0.001$; $\eta^2 = 0.17$; Table 1; Fig. 8A) and weighed less ($F_{1, 66} = 18.78$, $p < 0.0001$; $\eta^2 = 0.22$; Table 1; Fig. 8B) than males that continued to call. These males were also in poorer body condition than males that continued to call ($F_{1, 66} = 4.23$, $p = 0.04$; $\eta^2 = 0.06$; Table 1; Fig. 8C). Sufficient volumes of plasma for glucose analysis were available for 54 of the 68 males (i.e., most plasma was used for radioimmunoassay to quantify circulating hormone levels). Plasma glucose levels were not significantly different for males that stopped and continued to call in response to broadcast advertisement calls ($F_{1, 52} = 0.09$, $p = 0.76$; $\eta^2 = 0.002$; Table 1).

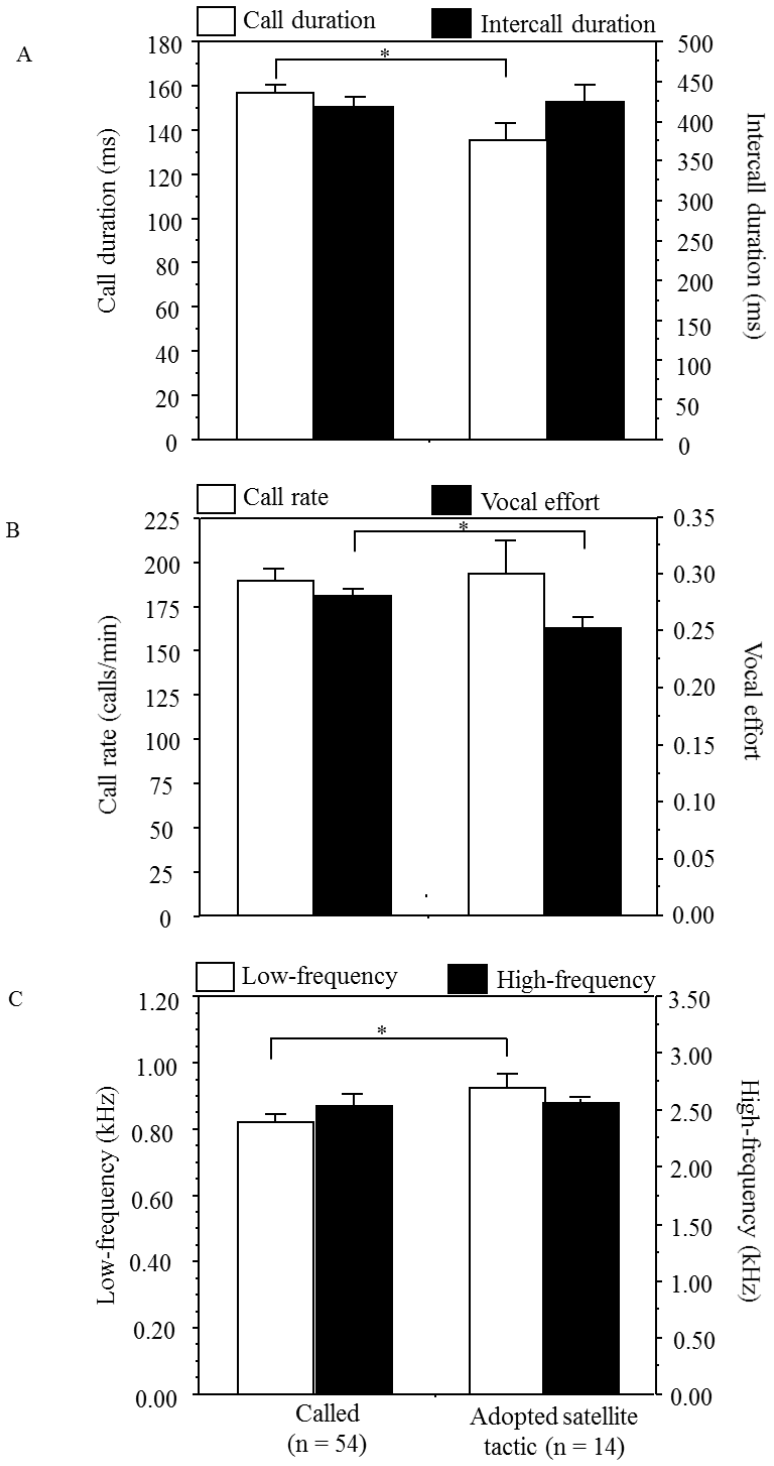

Figure 7. (A) Call and intercall duration, (B) call rate and vocal effort, and (C) low and high dominant carrier frequencies in males that called and males that adopted the satellite tactic in response to advertisement stimuli. Bar with asterisk indicates significant differences in vocal parameters. Whiskers indicate ±1 SE.

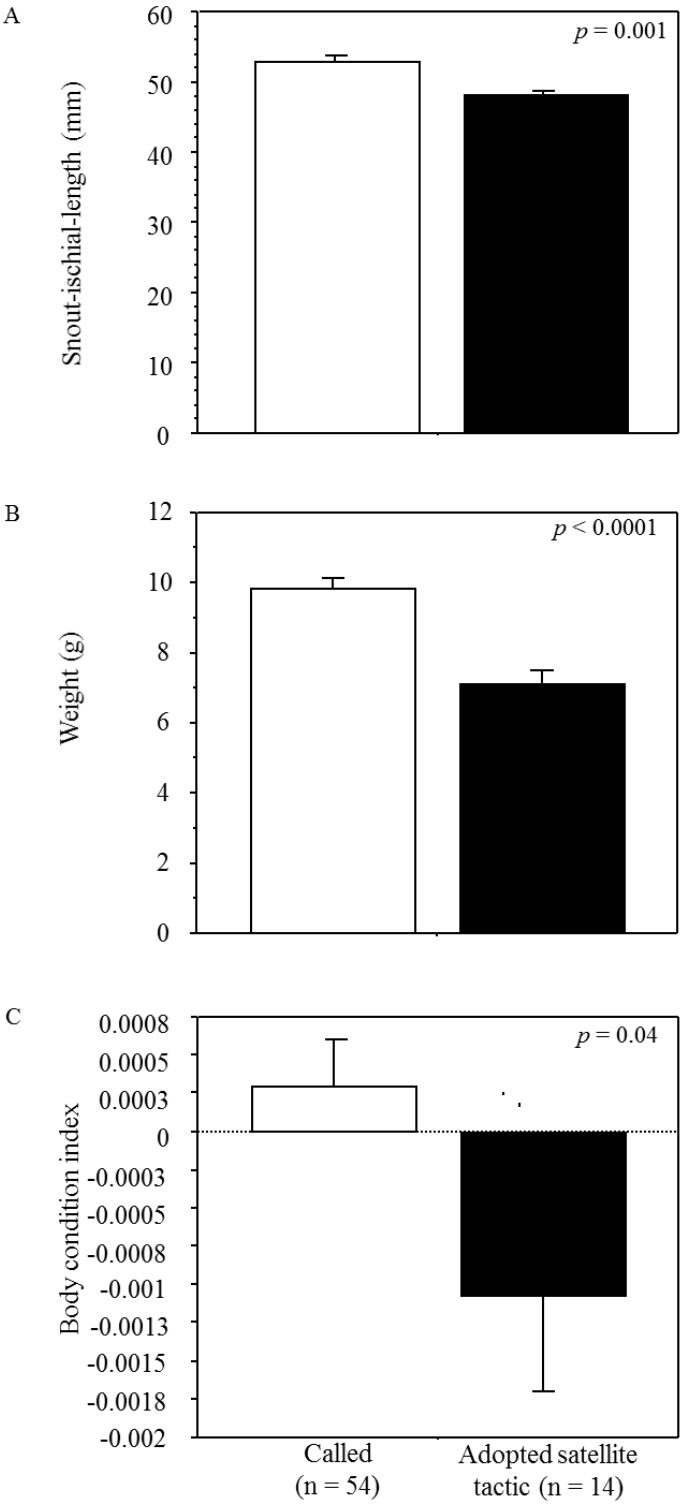

Figure 8. (A) Snout-ischial-length (SIL), (B) weight and (C) body condition in males that called and males that adopted the satellite tactic in response to broadcast advertisement calls. Whiskers indicate ±1 SE.

Table 1. ANOVA comparisons between males that continued to call and males that stopped calling for all measured variables. Bold indicates significance, alpha at 0.05

		Called Consistently	Stopped Calling	ANOVA Analysis	
Parameter	**N**	**Mean ± SE**	**Mean ± SE**	***F***	***p***
DHT (ng/ml)	63	31.77 ±1.42	28.17 ± 3.87	1.15	.29
T (ng/ml)	63	15.09 ± .80	13.44 ± 1.55	.94	.34
CORT (ng/ml)	63	2.91 ± .40	4.36 ± 1.07	2.43	.13
SIL (mm)	68	53.00 ± .66	48.14 ± .64	13.09	**.001**
Weight (g)	68	9.84 ± .31	7.08 ± .41	18.78	**< .0001**
Condition (index)	68	.00 ± .00	-.001 ± .00	4.23	**.04**
Plasma Glucose (mg/100ml)	53	112.84 ± 12.34	109.92 ± 25.11	.01	.91
Low carrier frequency (kHz)	67	.82 ± .02	.93 ± .04	4.06	**.05**
High carrier frequency (kHz)	67	2.57 ± .03	2.53 ± .11	.20	.65
Call duration (ms)	67	156.95 ± 3.52	135.83 ± 7.38	6.92	**.01**
Intercall duration (ms)	67	416.83 ± 11.15	423.27 ± 20.85	.07	.80
Call rate (calls/min)	67	188.73 ± 6.94	192.68 ± 18.93	.06	.82
Vocal effort	67	.28 ± .01	.25 ± .01	6.38	**.01**

Relationships among dependent variables

Estimates of all possible pairwise correlations among the measured variables are reported in Appendix A. Results indicated a significant positive correlation between body condition and circulating levels of DHT ($p = 0.01$) and T ($p = 0.03$) and a negative correlation between body condition and circulating levels of CORT ($p = 0.01$). Weight measurements were also positively correlated with circulating levels of T ($p = 0.03$) and negatively correlated with levels of CORT ($p = 0.04$). Though, body size was not correlated with circulating levels of androgens or CORT ($p > 0.2$). Body size was, however, positively correlated with plasma glucose levels ($p = 0.04$). In addition, body size and weight were negatively correlated with low-frequency ($p = 0.002$; $p = 0.002$, respectively) and high-frequency ($p = 0.007$; $p < 0.0001$, respectively) and positively correlated with call duration ($p = 0.01$; $p = 0.02$, respectively). Body condition was negatively correlated with high-frequency ($p = 0.04$). Circulating levels of DHT and T were not correlated with any acoustic parameters ($p > 0.3$ for all comparisons); however, circulating levels of CORT were positively correlated with call rate ($p = 0.005$).

Logistic regression analysis

Logistic regression was used to model male mating tactic expression in response to the advertisement call stimulus since the criterion variable is dichotomous (0=continued to call, 1=stopped calling). Predictor variables included SIL, body condition, CORT, high-frequency, intercall duration, call rate and vocal effort. Results indicated that the seven-predictor model afforded a statistically significant improvement over the constant-only model ($\chi^2_{(7)} = 32.91$, $p < 0.0001$). The Nagelkerke pseudo R^2 indicated that the model accounted for 60% (-2LL = 33.83) of the total variance, which suggests that the set of predictor variables discriminate between

males that continue to call and males that adopt satellite behavior in response to broadcast advertisement calls. Prediction success for the cases used in the development of the model was relatively high, with an overall prediction success of 88.7% and correct prediction rates of 95.9% for those males that continued to call and 61.5% for those males that adopted satellite behavior. The Hosmer and Lemeshow test indicated that the model accurately predicted the actual probabilities ($\chi^2_{(8)} = 4.97$, $p = 0.76$).

Table 2 presents the regression coefficients (B), Wald statistics, significance level, odds ratio [Exp (B)], and 95% confidence intervals (CI) for the odds ratios for each predictor. Based on the Wald test, body size (e.g., SIL) was ranked as the most significant predictor of tactic decisions in response to broadcast advertisements calls followed by high carrier frequency, condition and vocal effort. The negative B coefficients indicate that as males increase in body size and condition, and their high peak frequency and vocal effort increase, the probability of males adopting satellite behavior in response to male vocalizations decreases (Fig. 9A, B, C, & D) – this is after accounting for all other variables in the model.

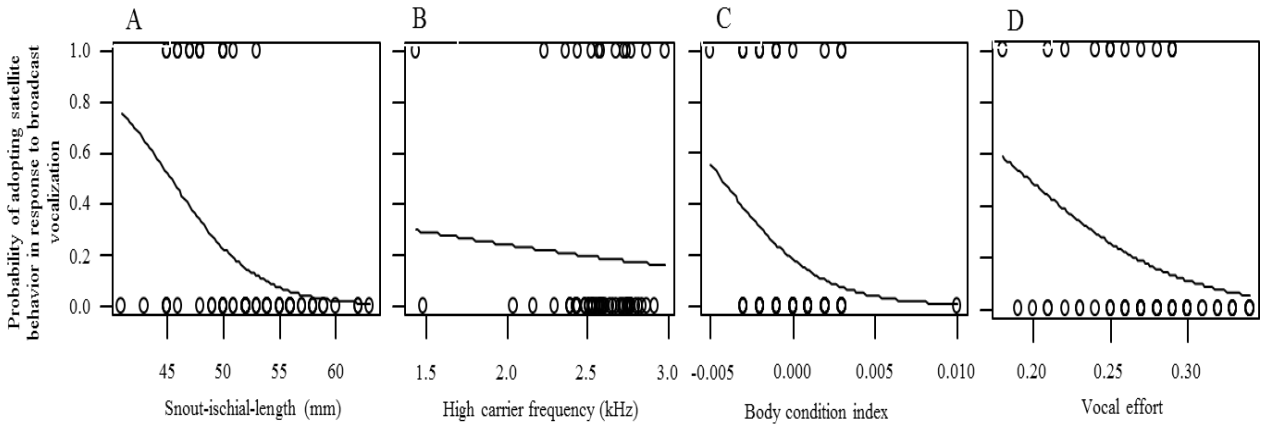

Figure 9. Results of the logistic regression analysis plotted with (A) snout-ischial-length, (B) high carrier frequency, (C) body condition, and (D) vocal effort on the x-axis and the probability of adopting satellite behavior on the y-axis.

Table 2. Results from a logistic regression analysis for dichotomous variables (0 = calling, 1 = adopted satellite behavior) with predictor variables SIL, condition, CORT, high frequency, intercall duration, call rate and vocal effort.

					95.0% CI for Exp(B)	
	B	**Wald**	**Significance**	**Exp(B)**	**Lower**	**Upper**
SIL (mm)	-0.36	9.00	**0.003**	0.70	0.55	0.88
Condition	$-4.2e^{+2}$	4.37	**0.04**	$7.8e^{-184}$	0.00	$1.6e^{-4}$
CORT (ng/mL)	0.26	2.70	0.10	1.30	0.95	1.77
High Frequency (kHz)	-4.01	4.66	**0.03**	0.02	0.00	0.69
Intercall Duration (ms)	-0.01	1.84	0.17	0.99	0.98	1.01
Call Rate (calls/min)	-0.02	2.30	0.13	0.98	0.97	1.01
Vocal Effort	$-3.2e^{+1}$	4.26	**0.04**	$8.76e^{-15}$	0.00	0.19

B values are the estimated unstandardized regression coefficients which indicate the amount of change expected in the log odds when there is a 1-unit change in the predictor variables with all other variables held constant. The Wald statistic tests the statistical significance of each coefficient (B). The Exp(B) is the odds ratio and CI are the confidence intervals for the odds ratio.
Bold indicates significance at alpha 0.05.
N = 62

Discussion

Despite previous results indicating that satellite *H. cinerea* males possess higher circulating levels of CORT and lower levels of androgens relative to calling males (Leary and Harris, 2013) and elevated levels of CORT are causally associated with satellite tactic expression in this species (see Chapter 1), the present study found no evidence that CORT or androgens predicted mating tactic expression in response to broadcast advertisement calls. Rather, the results indicate that body size, condition, and vocal attractiveness are the best predictors of satellite tactic expression suggesting that multiple factors contribute to mating tactic decisions in this species.

Results from this study provide some support for the "relative attractiveness hypothesis", (e.g., that tactic expression is influenced by the attractiveness of nearby calling males, see Waltz, 1982; Arak, 1988). For example, males that adopted satellite behavior in response to broadcast advertisement calls produced vocalizations (prior to playback experiments) that were shorter in duration than males that continued to call. Producing advertisement calls of short duration can potentially have a significant impact on the chances of mate acquisition because female *H. cinerea* show a preference for calls of long duration (Gerhardt, 1987). However, the advertisement calls of the two groups of males did not differ in call rate, which has also been implicated as an important acoustic parameter in mate choice by females (Gerhardt and Huber, 2002; Humfeld, 2008). Conversely, males that adopted satellite behavior in response to broadcast advertisement calls produced calls with an overall lower vocal effort indicating that energetic investment in vocalization was on average lower than that of males that continued to call.

There was some evidence that the dominant carrier frequencies of advertisement calls predict male responses to broadcast advertisement calls. For example, males that adopted

satellite behavior in response to advertisement call stimuli produced calls with an average low-frequency peak that was slightly higher (mean = 0.93 kHz) than the mean peak frequency of males that continued to call (mean = 0.82 kHz). Whether this difference translates into differences in vocal attractiveness is not entirely clear. Although female *H. cinerea* generally prefer call carrier frequencies that approximate the mean of the population (i.e., are under stabilizing selection, *reviewed* by Gerhardt and Huber, 2002), such preferences can be reduced by manipulating the sound intensity (SPL) of male advertisement calls (Gerhardt, 1982; 1987; 1991). For example, discrimination against lower-than-average low-frequency alternatives decreases with increasing sound intensity (65 – 85 dB SPL; Gerhardt, 1987); however, discrimination against higher-than-average low-frequency alternatives is maintained at high sound intensities (Gerhardt, 1987). In natural choruses, the sound intensity of signals produced by calling males and satellites that vocalize after removal of the host have similar intensities (an average of 88 dB SPL; Humfeld, 2008). Thus, producing calls with a low-frequency peak that is higher than the mean can potentially influence mate choice by females and be a significant predictor of satellite mating tactic expression.

The results indicate that the response of males to broadcast advertisement calls can be predicted by high carrier frequency, though this is after accounting for variation in body size and carrier frequency is a correlate of body size. Carrier frequencies of male advertisement calls can be constrained by body size and its influence on sound producing structures (i.e., larynx; Gerhardt and Huber, 2002). Thus, carrier frequencies are assessed in vocal contests with rival males as indicators of body size and fighting ability (Davies and Halliday, 1978; Arak, 1983; Ramer et al., 1983; Robertson, 1986; Wagner, 1992); larger, more competitive males, produce advertisement calls with lower frequency bands relative to smaller males (Davies and Halliday,

1978). Mutual assessment of body size can be particularly important when deciding whether to engage in a contest with a conspecific or avoid such costly interactions (Wagner, 1992). Because satellites are generally smaller and in poorer body condition than calling males (Forester and Lykens, 1986; Eggert and Guyetant, 2003; Leary et al., 2005; Castellano et al., 2009; Brepson et al., 2012), males adopting satellite behavior potentially choose to parasitize larger males to achieve mate acquisition, rather than engage in contests that could result in injuries (Berec and Bajgar, 2011).

Logistic regression analysis indicated that size was the most robust predictor of tactic expression after accounting for the other model variables. This result may be an effect of the influence of body size on vocal attractiveness, body condition, and circulating hormone levels. For instance, body size and condition can affect the magnitude of glucocorticoid secretion in response to stressors (Dunlap and Wingfield, 1995; Kitaysky et al., 1999; Jessop et al., 2002; Leary, 2014; *reviewed* by Moore and Jessop, 2003; Husak and Moore, 2008). For example, there is a size-related effect of acoustic signals on stress hormone production in male *H. cinerea* with smaller males showing a greater increase in CORT production and, generally, a decrease in DHT levels in response to broadcast advertisement calls (Leary, 2014). In the present study, while body size was not correlated with circulating levels of CORT, there was a detectable negative correlation between body condition and CORT levels. While males that adopted satellite behavior did not have significantly higher levels of CORT compared to males that continued to call, they were in poorer body condition. Thus, although CORT level did not predict mating tactic decisions in response to broadcast advertisement calls, correlates of CORT, such as body condition, did predict responses to broadcast advertisement calls.

I also explored whether males that called and males that adopted satellite behavior exhibited variation in plasma glucose levels. The impetus for this analysis stemmed from the gluconeogenetic effects of CORT on hepatic metabolism that mobilizes glucose (Exton, 1979). In anurans, the gluconeogenic effects of CORT are expected to mobilize glucose to meet the energetic demands of acoustic signaling (Emerson, 2001); calling in anurans is one of the most energetically expensive behaviors known in ectothermic vertebrates (Taigen and Wells, 1985). The EHV model proposes that CORT reaches a threshold level when energy reserves are depleted that negatively affects androgen production and, hence, calling behavior (Emerson, 2001). While males that adopted satellite behavior were in poorer body condition and produced calls indicative of lower energetic investment compared to males that called, plasma glucose levels were not different between the two groups of males. Furthermore, circulating levels of CORT and plasma glucose levels were not significantly correlated. These results suggest that circulating levels of CORT maintain constant levels of blood glucose in individuals that vary in body condition. There was, however, a positive association between size and plasma glucose levels with larger males exhibiting higher plasma glucose levels, suggesting that smaller males on average may have less energy available for signaling.

***Cause versus consequence*?**

The overall aim of this project was to address the concerns set forth by Oliveira et al. (2008) on the bidirectionality of hormone-behavior relationships and interpretation of varying hormone levels among males exhibiting alternative mating tactics (i.e., application of the RPH). The present study investigated the prediction that circulating levels of CORT would be higher and androgen levels would be lower in males adopting the satellite tactic; a prediction that

stemmed from satellite male *H. cinerea* in natural choruses possessing higher CORT and lower androgen levels than calling males (Leary and Harris, 2013). However, unexpectedly, the results indicated that hormone level did not predict male mating tactic expression in response to broadcast advertisement calls.

These results indicate that the relationship between hormone level and mating tactic expression is much more complicated than previously thought. For instance, we know that social interactions between males can stimulate the production of CORT (Leary, 2014) that, in turn, can decrease vocal attractiveness and increase the probability of satellite behavior (see Chapter 1). Furthermore, satellite *H. cinerea* that vocalize after the removal of the host male produce unattractive calls that are short duration and have high carrier frequencies (Humfeld, 2008). Despite the negative effects of experimentally elevated CORT on vocal attractiveness, there was no correlation between CORT and the magnitude of vocal parameters. The lack of a detectable significant correlation may be because calling males possess a very narrow range of CORT levels (mean = 3.21, range = 0.03 – 12.9, ng/ml). In contrast, calling and satellite males in natural choruses have a much wider range of circulating CORT (see Leary and Harris, 2013). This suggests that CORT may be such a powerful mediator of mating tactic expression, that all males with high CORT and low androgens may adopt satellite behavior in natural choruses. For instance, 92% of 12 calling males injected with CORT adopted satellite behavior within 75-min post injection (see Chapter 1). Nonetheless, the results indicate that the attractiveness and size of a male relative to conspecifics in the social-acoustic environment can also influence tactic decisions independently of variation in hormone levels.

Overall, these results suggest that differences in circulating hormone levels in satellites and callers in natural choruses (see Leary and Harris, 2013) may be either a cause or

consequence of tactic expression. For example, while social interactions can stimulate CORT production (Leary, 2014) and increase the probability of satellite tactic expression (see Chapter 1), vocally unattractive and/or smaller males may adopt satellite behavior in response to the social-acoustic environment. Adoption of satellite behavior may, in turn, result in changes in circulating hormone levels during interactions with calling host males.

Conclusions

Despite considerable effort aimed at understanding the hormonal basis for alternative mating tactics, relatively few studies have demonstrated that differences in circulating hormone levels among plastic reproductive phenotypes, when they exist, are causally related to the adoption of alternative reproductive behaviors (Oliveira et al., 2008). For example, differences in circulating hormone levels could arise because of changes in the social dynamics of interacting individuals after tactic adoption. These issues are rooted in the bi-directionality of hormone-behavior relationships (Oliveira et al., 2008) and critical to understanding the potential mechanisms regulating the expression and maintenance of variation in reproductive behavior in natural populations. Here I showed in male green treefrogs that even when differences in hormone levels for the behavioral phenotypes are known to be causally associated with tactic expression, that changes in the social environment can elicit changes in mating tactic expression that are not predicted by circulating hormone levels. That is, there may not be a clear "cause versus consequence" dichotomy associated with hormonal differences in behavioral phenotypes because multiple factors can mediate changes in tactic expression and circulating hormone levels. This work emphasizes that multiple approaches are required to understand the potential

suite of factors that can influence mating tactic expression and to address whether differences in behavioral phenotypes are a cause or consequence of mating tactic expression

CHAPTER THREE

THE ENDOCRINE DYNAMICS OF ALTERNATIVE MATING BEHAVIORS IN GREEN TREEFROGS: STRESS AND SEX STEROIDS PREDICT CALLING BEHAVIOR IN SATELLITE MALES

Abstract: Intraspecific competition has resulted in the evolution of a diverse array of male armaments that effectively increase reproductive success in the bearer by decreasing the mating prospects of rival males. Individuals that cannot effectively compete with males possessing superior weapons often resort to adopting alternative mating tactics. In male green treefrogs, *Hyla cinerea*, armaments consist of acoustic signals that elicit elevations in circulating stress hormone levels (corticosterone, CORT) and a reduction in androgen levels. Such changes in circulating hormones increase the probability of non-calling "satellite" mating tactics but may also occur as a consequence of mating tactic expression. I hypothesized that acoustically-induced alterations to the endocrine physiology of receivers benefits male signalers by negatively affecting the attractiveness of satellite male courtship signals and the propensity to vocalize. To address this hypothesis, I removed calling "host" males from the vicinity of associated satellite males and examined the vocal responses and hormone levels of satellite males. Results revealed that satellite males have higher circulating levels of CORT, lower circulating levels of androgens, are smaller and in poorer condition than calling males. Upon removal of the calling host male, 74% of satellite males began to vocalize and those males produced calls that were

significantly shorter in duration and lower in vocal effort than host males. Conversely, 26% of satellite males did not call subsequent to removal of the calling host male. These males had significantly lower levels of circulating androgens relative to host males and satellite males that began to call after host removal. These results suggest that acoustic modulation of circulating hormone levels can impose costs on males by decreasing vocal attractiveness and suppressing courtship behavior.

Introduction

Competition among males for access to mates can be intense, especially in highly male-biased breeding aggregations (Clutton-Brock and Vincent, 1991; Clutton-Brock and Parker, 1992). As a result, males have evolved an array of armaments to effectively maximize mating opportunities (Andersson, 1994; Berglund et al., 1996; Emlen, 2008; 2014). Asymmetries in male armaments often determine the outcome of animal contests (Parker, 197; Smith and Parker, 1976; Smith, 1982: Briffa and Sneddon, 2006); for example, male shore crabs (*Carcinus maenas*) with longer chelae (i.e., pincer-like claws) are more likely to win contests than crabs with smaller chelae (Sneddon et al., 1997). Because engaging in contests with dominant rival males is potentially costly (i.e., increases the risk of injury and predation; *reviewed* by Briffa and Sneddon, 2006), competitively inferior males may adopt alternative mating tactics (AMTs) to minimize such risks and optimize their chances of mate acquisition (Dominey, 1984; Andersson, 1994; Gross, 1996; Brockmann, 2001; Shuster, 2010; Taborsky et al., 2008).

The mating systems of various invertebrate and vertebrate species (i.e., horned beetle, *Onthophagus acuminatus*, Emlen, 1997; the ruff, *Philomachus pugnax*, Lank et al., 1995; blue gill sunfish, *Lepomis macrochirus*, Gross and Charnov, 1980) often consist of at least two

conditional AMTs (Dominey, 1984; Andersson, 1994; Gross, 1996; Brockmann, 2001; Taborsky et al., 2008; Shuster, 2010). Conditional switching between the dominant and alternative mating tactic is predicted to be based on perceived fitness trade-offs under frequency- and status-dependent selection (Gross, 1996). Status-dependent selection, in particular, occurs when tactic expression is based on relative competitive ability (Gross, 1996) which can be dependent on numerous factors including age, body size and condition. In horseshoe crabs (*Limulus polyphemus*), for example, males in poor body condition adopt a satellite mating tactic and achieve reproductive success via sperm competition with copulating males (Brockmann, 2002). Fundamentally, the "status" of a male dictates the threshold at which the dominant or alternative tactic best maximizes mating success (Gross, 1996).

Sex and stress steroids are important indicators of male status and competitive ability (Creel, 2001; Oliveira et al., 2002; Oliveira, 2004; 2005; Miles et al., 2007). For example, gonadal steroids (e.g., androgens) mediate the expression and development of male courtship signals (e.g., tree lizards, *Urosaurus ornatus*, Hews et al., 1994; Hews and Moore, 1995; Hews et al., 1996; dark-eyed junco, *Junco hyemalis*, Ketterson et al., 1992; Enstrom et al., 1997) and are also often positively correlated with aggression and territoriality (Wingfield et al., 1990). Furthermore, variation in circulating androgen levels have activational effects on mating tactic expression (*reviewed* by Oliveira et al., 2008; Moore, 1991; Thompson and Moore, 1992; Moore et al., 1998). For example, testosterone implants in the blue throated morph of side-blotched lizards (*Uta stansburiana*) results in transitions from a non-territorial to territorial phenotype in which they increase home range size and secure access to more females (Sinervo et al., 2000). In contrast, elevated stress hormones (e.g., glucocorticoids) often suppress dominant courtship behavior and can promote the expression of alternative mating tactics (Saino et al., 2002;

Buchanan et al., 2003; Spencer et al., 2003; 2004; Leary et al., 2006a; 2006b; Roulin et al., 2008; Leary and Knapp, 2014), potentially via negative effects on sex steroid production (Leary and Knapp, 2014; Moore et al., 2015).

Changes in glucocorticoid and androgen levels are associated with male contests (*reviewed* by Harding, 1981; Creel, 2001; DeVries et al., 2003; Creel et al., 2013). For example, male contest losers often have higher glucocorticoid levels and lower androgen levels than contest winners (Bronson and Eleftheriou, 1964; Louch and Higginbotham, 1967; Manogue et al., 1975; Leary, 2014). In male green treefrogs, agonistic acoustic signals stimulate production of the stress hormone corticosterone (CORT) and decrease production of androgens in rival males (Leary, 2014). High circulating levels of CORT in male *H. cinerea* compromise vocal attractiveness and increase the probability of alternative non-calling "satellite" behavior (see Chapter 1). Non-calling satellite male *H. cinerea* crouch near calling "host" males and attempt to intercept females attracted to the host male's vocalizations (Perrill et al., 1978; Perrill et al., 1982; Perrill and Magier, 1988; Humfeld, 2008; Leary and Harris, 2013; see Chapters 1 and 2). Satellite males in natural choruses possess higher circulating levels of CORT and lower levels of androgens compared to calling host males (Leary and Harris, 2013). Elevated CORT appears to be of primary importance in mediating satellite tactic expression in *H. cinerea* because CORT injections increase the probability of satellite behavior independently of changes in circulating androgen levels (see Chapter 1). Furthermore, the effects of CORT administration on mating tactic expression are dependent on the social acoustic environment; for instance, elevated CORT predominately elicits satellite tactic expression during simulated male-male vocal interactions (see Chapter 1).

Consistent with a context-dependent effect of elevated CORT on mating tactic expression, previous studies with anurans (including *H. cinerea*) have shown that the removal of the calling host male from a satellite-caller association elicits calling behavior in satellite males (Perrill et al., 1982; Arak, 1988; Perrill and Magier, 1988; Humfeld, 2008). However, such studies often report considerable variation in the probability that a male exhibiting satellite behavior will adopt calling behavior after removal of calling host males (Leary et al., 2006a). For instance, satellite male natterjack toads (*Bufo calamita*) adopted calling behavior in 20% of 10 host removal experiments (Arak, 1988). Similarly, in *H. cinerea*, 18% of 17 satellite male *H. cinerea* adopted calling behavior after removal of the host male (Perrill et al, 1978). Why some males call and others do not after removal of the host is unclear.

Here, I examine whether circulating levels of CORT and androgens predict calling behavior in satellite male *H. cinerea* subsequent to removal of the host male. Although elevated CORT level increases the probability of satellite tactic expression in this species (see Chapter 1), elevated CORT levels are not necessary to elicit changes in mating tactic expression (see Chapter 2). For example, males with relatively low CORT levels may adopt satellite behavior in response to simulated territorial intrusions (i.e., broadcast advertisement calls), suggesting that the endocrine status and social environment can both play primary roles in mediating tactic expression (see Chapter 2). However, satellite males in natural choruses possess higher CORT levels and lower androgen levels than calling males (Leary and Harris, 2013), suggesting that the endocrine physiology of males that adopt a satellite mating tactic is potentially altered during vocal interactions with the dominant calling host male, regardless of the endocrine status of males when satellite behavior was initially adopted. That is, high CORT levels and low androgen levels can be a cause or consequence of mating tactic expression in this species (see Chapter 2).

I predicted that variation in the endocrine status of satellite male *H. cinerea*, stemming from the effects of acoustic armaments during male-male vocal interactions, accounts for the variation in male calling behavior subsequent to removal of the calling host male. Specifically, I predicted that satellite males with elevated CORT level call upon removal of the calling host male (reflecting context-dependency of CORT on calling behavior) but that males with low androgens do not call subsequent to host removal. I also examined the relationship between circulating hormone levels and vocal attractiveness. CORT administration has negative effects on acoustic parameters that are important in mate choice in this species (i.e., call duration, vocal effort; see Chapter 1), but it is not clear whether these effects translate into variation in vocalization of males in natural choruses. For example, there was no detectable relationship between CORT level and vocal effort in calling males in natural choruses (see Chapter 2).

Lastly, I also examined how body size and condition contribute to variation in male calling behavior because both parameters are linked to satellite tactic expression and vocal parameters. For example, body size is often negatively correlated with dominant carrier frequencies (Davies and Halliday, 1978; see Chapter 2) and satellite males are consistently smaller than host males (Forester and Lykens, 1986; Eggert and Guyetant, 2003; Leary et al., 2005; Humfeld, 2008; Castellano et al., 2009; Berec and Bajgar, 2011; Brepson et al., 2012). Furthermore, both body size and condition can influence circulating hormone levels (Dunlap and Wingfield, 1995; Kitaysky et al., 1999; Jessop et al., 2002; Leary, 2014; *reviewed* by Moore and Jessop, 2003; Husak and Moore, 2008). Therefore, I explored the influence of size and condition on variation in calling behavior, vocal attractiveness, and circulating hormone levels.

Methods

General experimental design and satellite-host manipulation experiments

Satellite-caller associations were studied in natural choruses at the University of Mississippi Field Station (Lafayette County, MS, USA) throughout peak hours of reproductive activity (~2000-2200 hour) during the 2014 and 2015 breeding seasons. Satellite-caller associations were identified (using low-powered headlamps to minimize disturbance) and observed for approximately five minutes to ensure that the association was stable (i.e., that satellite males did not begin to vocalize during the focal observation period). Body temperatures of callers and associated satellites were then measured to the nearest 0.1ºC (range 22.0 – 26.0ºC) and a series of approximately 30 calls were recorded from the calling host male using a Marantz PMD 222 cassette recorder equipped with a Sennheiser directional condenser microphone (Model ME-66). Subsequently, the calling host male was removed by hand from the vicinity of the associated satellite male and the satellite's behavior was subsequently observed for a maximum of 20 min. Blood samples were obtained via cardiac puncture from calling host males immediately following capture and were obtained from satellite males immediately following focal observations (subsequent to host removal). Snout-ischial-length (SIL) and weight were then measured following acquisition of blood samples. Acquisition of blood typically occurred in less than 4 min, and measures of SIL and weight were obtained in under 10 min (see Chapter 2).

Following removal of associated calling host males, satellites either 1) began to vocalize or 2) continued non-calling satellite behavior (see Perrill et al., 1978; Arak, 1988). If the satellite male began to vocalize during the focal period, a series of at least 30 vocalizations were recorded. Males were then marked on the venter using a portable tattoo device (Tattoo-A-Pet, Fort Lauderdale, FL, USA) prior to release at the site of capture for future identification. Data

was obtained from 35 satellites and 37 callers; sample sizes for the behavioral phenotypes were not equal because two satellite males were associated with two calling host males.

Body condition calculations

Body condition estimates were used as proxies for energy reserves. Body condition was calculated using the residual values from a linear regression of the cubed root body weight on SIL divided by SIL (see Baker, 1992; Leary et al., 2008).

Vocal analysis

Calls were analyzed using Raven Pro acoustics software (Cornell Bioacoustics Laboratory). Acoustic parameters known to be important in mate choice by females (Gerhardt, 1974; 1978b; 1982; 1987; 1991; Gerhardt et al., 1990; Gerhardt and Huber, 2002; Humfeld, 2008) were measured from the middle portion of 5-10 consecutive calls; these parameters included call duration (ms), intercall duration (ms), call rate (calls/min), and peak low and high call carrier frequencies (kHz). I then calculated vocal effort (e.g., proportion of time spent calling) by dividing call duration by the sum of call duration and intercall duration. Vocal effort was used as a proxy for energy expended during vocalization because the proportion of time spent vocalizing contributes to overall acoustic energy and is positively correlated with aerobic metabolic rate (see Taigen and Wells, 1985; Pough et al., 1992; Prestwich et al., 1994). Amplitude (dB) was not measured since accurate sound intensity was difficult to obtain in many cases (i.e., males often call from dense thickets of vegetation that prevent consistent positioning of the amplitude meter) and previous studies showed that satellites produce calls with similar intensity as other calling males (Leary et al., 2006a; Humfeld, 2008).

Sample preparation, column chromatography, and radioimmunoassay procedures

Hormone separation and quantification of hormone concentrations followed the methods described in Leary et al. (2004). Briefly, 30µl of each individual plasma sample was allocated into separate extraction tubes and incubated overnight with radiolabeled hormone (PerkinElmer, Inc. Hebron, Kentucky). Steroids were then extracted from the plasma using diethyl ether and subsequently dried under nitrogen gas at 40ºC. Dried samples were then resuspended in 10% ethyl acetate in iso-octane and loaded onto diatomaceous earth columns containing a 3:1 diatomaceous earth:distilled water "glycol trap" and a 1:1 propanediol:ethylene glycol mixture. Mixtures of 10%, 20%, and 52% ethyl acetate in iso-octane were then used to collect DHT, T, and CORT, respectively. Fractions were dried under nitrogen gas and resuspended in phosphate buffered saline containing 0.3% gelatin for use in radioimmunoassay. T antibody was purchased from Fitzgerald Industries International, Inc. (Acton, Massachusetts) and used to measure both DHT and T. The CORT antibody was purchased from MP Biomedicals, LLC (Solon, Ohio). All samples were assayed in duplicate.

Plasma samples were analyzed for DHT, T and CORT levels in 2 assays. Mean intra-assay coefficients of variation for DHT, T, and CORT were 3.7%, 5.8%, and 6.0%, respectively, based on 4 standards run with each assay. Inter-assay coefficients of variation for DHT, T, and CORT were 1.1%, 1.0%, and 1.4%, respectively.

Statistical analysis

Body temperatures can alter various attributes of male advertisement calls in anurans (Gerhardt and Huber, 2002). Thus, a simple linear regression analysis was performed on all measured acoustic parameters against body temperature. Vocal parameters that were

significantly correlated with temperature (i.e., call duration and intercall duration) were corrected to the mean recording temperature (24ºC) by calculating the residual values obtained from the linear regression and using the linear equations from the regression lines to calculate the adjusted value (Leary et al., 2008).

A one-way multivariate analysis of variance (MANOVA) test was used to compare circulating levels of CORT and androgens, body size and condition in calling host males and satellite males. The assumption of normality was assessed by performing a Shapiro-Wilk test and existence of univariate and/or multivariate outliers were assessed using a boxplot and Mahalanobis distance (Meyers et al., 2006). A Box's M test was used to test the equality of covariance between groups; if the assumption of homogeneity of covariance was violated, the reported *p* value is from the Pillai's Trace multivariate method, which is considered the most robust to deviations from the assumption of equal variances (Quinn and Keough, 2002). Univariate ANOVAs were conducted on each dependent variable separately to determine the location of the statistically significant multivariate effect. A Levene's test was used to test the assumption of homogeneity of variance for all univariate ANOVAs. If there was a significant difference between groups, a post hoc test was conducted to determine differences between callers and the two groups of satellite males (e.g., satellites that called and did not call subsequent to removal of the calling host male). I chose to use a Games-Howell non-parametric post-hoc test that is robust to deviations from normality and does not rely on equal variances or sample sizes (Games and Howell, 1976).

Of the satellites males that began to vocalize subsequent to removal of the calling host male, I ran a MANOVA with each acoustic parameter to compare their vocalizations to the vocalizations of the host male. I previously showed that elevations in circulating levels of CORT

can negatively affect characteristics of advertisement calls (see Chapter 1), yet whether these effects of CORT on vocal parameters translate into variation in natural choruses is unclear because circulating CORT is uncorrelated with acoustic parameters within calling males (see Chapter 2). Hence, I conducted a multiple regression to examine associations between circulating hormone levels (i.e., androgens and CORT) and vocal parameters for host and satellite males. A Durbin-Watson test was used to test the assumption of independence of observations (e.g., autocorrelation; McAuliffe, 2005). Linearity and homoscedasticity was assessed by visual inspection of a plot of studentized residual versus unstandardized predicted values. Multicollinearity was then tested by estimating variance inflation factors (VIF) which assess the degree of correlation among variables (O'Brien, 2007); a VIF > 10 is indicative of robust multicollinearity (Mason et al., 1989). However, VIFs remained below 10 and thus none of the variables were removed from the model.

Results

After removal of the host, 26/35 (74%) of the satellite males began to vocalize while 9/35 (26%) satellite males remained silent. The latencies at which satellite males began to vocalize after host removal ranged from 50 up to 840 sec (mean = 357s ± 42.40; Fig. 1); satellites were designated as non-callers if they did not emit a vocalization within 20 min of host removal. Two of nine satellite males that remained silent oriented and moved towards a secondary caller after removal of the primary host. When the secondary callers were removed, these males remained silent for the duration of the experiment. Conversely, seven of the nine satellites remained silent and stationary for the entirety of the experiment.

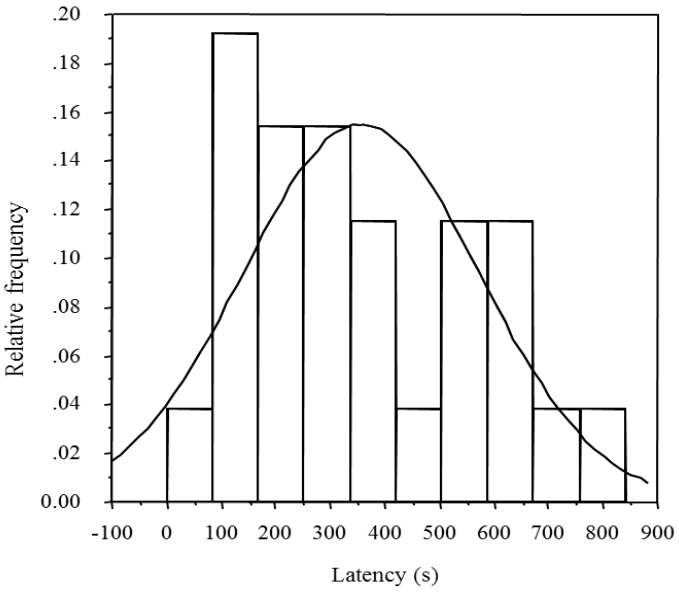

Figure 1. Histogram showing the distribution of vocal latencies in satellite males after host removal.

Radioimmunoassay results

Sufficient blood samples for radioimmunoassay (< 0.01 mL) were not obtained from 12 males and hormonal data for 6 males were eliminated because blood samples took longer than 4 min to acquire, after which there is a detectable increase in CORT ($r^2_{1,58} = 0.34$, $p = 0.009$) (see also Chapter 2). Thus, I obtained hormone data from 27 calling males and 27 satellite males (n = 19 satellites that called, n = 8 satellites that did not call).

The three categories of males possessed significantly different circulating hormone levels (Pillai's Trace = 0.71; $F_{6,100} = 3.02$, $p = 0.009$; $\eta^2 = 0.15$). Univariate ANOVAs showed that circulating levels of DHT differed between the three categories of males ($F_{2,51} = 5.87$, $p = 0.005$; $\eta^2 = 0.19$). DHT levels of satellite males that began to vocalize after host removal did not differ from calling host males (Games-Howell: $p = 0.19$; Fig 2). However, satellites that resumed non-calling behavior after removal of the host had significantly lower levels of DHT relative to hosts (Games-Howell: $p < 0.0001$; Fig. 2) and satellite males that began to vocalize (Games-Howell: p

= 0.02; Fig. 2). Conversely, circulating levels of T, overall, were not significantly different between the three categories of males ($F_{2,51} = 2.57$, $p = 0.09$; $\eta^2 = 0.09$; Fig. 2); however, satellites that did not call after removal of the calling host male possessed significantly lower levels of circulating T relative to host males (Games-Howell: $p = 0.007$; Fig 2).

Circulating levels of CORT were significantly different between the three categories of males ($F_{2,51} = 3.78$, $p = 0.03$; $\eta^2 = 0.13$). Satellite males that began to vocalize after removal of the host had marginally higher circulating levels of CORT relative to host males (Games-Howell: $p = 0.06$; Fig. 1); however, there were no differences in circulating levels of CORT between satellites that did and did not call after removal of the host (Games-Howell: $p = 0.59$; Fig. 1). Satellite males that did not call and host males did not differ with respect to circulating CORT levels (Games-Howell: $p = 0.72$; Fig. 1).

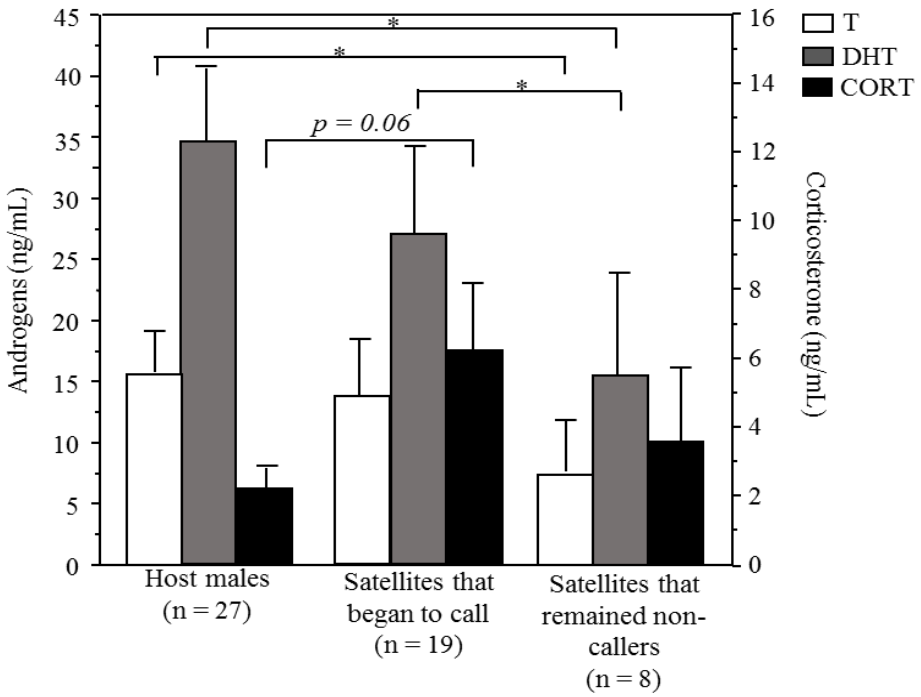

Figure 2. Bar graph depicting the average dihydrotestosterone (DHT), testosterone (T), and corticosterone (CORT) level of host males and satellite males that did and did not call after host removal. Bar with asterisk indicates significant differences in hormone levels. Whiskers indicate ±1 SE.

Body size, weight and condition

The three categories of males differed significantly in body size, weight and condition Pillai's Trace = 0.43; $F_{6,136} = 6.29$, $p < 0.0001$; $\eta^2 = 0.22$). Follow-up univariate ANOVA's showed that the three categories of males differed in SIL ($F_{2,69} = 17.56$, $p < 0.0001$; $\eta^2 = 0.34$), weight ($F_{2,69} = 20.90$, $p < 0.0001$; $\eta^2 = 0.38$) and body condition ($F_{2,69} = 4.03$, $p = 0.02$; $\eta^2 = 0.11$). Satellite males that called and satellite males that remained silent after host removal did not differ in SIL (Games-Howell: $p = 0.35$: Fig. 3A). However, both satellites that called and remained silent were smaller than host males (Games-Howell: $p < 0.01$ for both comparisons; Fig. 3A). Satellite males that called and satellite males that remained silent after host removal did not differ in weight (Games-Howell: $p = 0.83$; Fig. 3B), but both groups weighed significantly less than host males (Games-Howell: $p < 0.01$ for both comparisons; Fig. 3B). Lastly, Satellite males that called and satellite males that remained silent after host removal did not differ in body condition (Games-Howell: $p = 0.79$; Fig. 3C), but both groups were in significantly poorer body condition than host males (Games-Howell: $p < 0.01$ for both comparisons; Fig. 3C).

Relative attractiveness of advertisement calls

Host and satellite males that called produced advertisement calls with significantly different characteristics (Pillai's Trace = 0.52; $F_{6,53} = 9.61$, $p < 0.0001$; $\eta^2 = 0.52$). Univariate ANOVAs indicated that satellite males, who began to vocalize after host removal, produced shorter duration calls relative to host males ($F_{1,61} = 16.66$, $p < 0.0001$; $\eta^2 = 0.22$; Fig. 4A) as well as calls with a longer intercall duration ($F_{1,61} = 13.50$, $p = 0.001$; $\eta^2 = 0.19$; Fig. 4A). Furthermore, the two categories of males did not exhibit differences in call rate ($F_{1,61} = 0.58$, $p = 0.45$; $\eta^2 = 0.01$; Fig. 4B), but satellite males produced calls with lower overall vocal effort

relative to host males ($F_{1,61} = 25.57$, $p < 0.0001$; $\eta^2 = 0.31$; Fig. 4B). Lastly, satellite males produced advertisement calls with a higher low-frequency peak relative to host males ($F_{1,58} = 17.11$, $p < 0001$; $\eta^2 = 0.23$; Fig. 4C), but the high-frequency peak of advertisement calls was not significantly different between ($F_{1,58} = 0.36$, $p = 0.55$; $\eta^2 = 0.01$; Fig. 4C). Note that data for the low and high peak frequency measures of advertisement calls were not obtained for three individuals because of background (chorus) noise associated with recordings.

Associations between hormone level and vocal attractiveness

A multiple regression was then run to determine if DHT, T, and CORT accurately predicted characteristics of male advertisement calls. The multiple regression model marginally predicted call duration ($F_{3,42} = 2.59$, $p = 0.06$; *adj*. $r^2 = 0.10$; Table 1). Circulating levels of DHT and T were not statistically significant predictors of call duration ($p = 0.18$; $p = 0.83$, respectively); however, circulating levels of CORT negatively predicted call duration ($p = 0.05$; Fig. 5A). Conversely, the multiple regression model did not significantly predict intercall duration ($F_{3,42} = 1.88$, $p = 0.15$; *adj*. $r^2 = 0.06$; Table 1).

The multiple regression model significantly predicted call rate ($F_{3,42} = 3.49$, $p = 0.02$; *adj*. $r^2 = 0.14$; Table 1). Circulating levels of DHT and T were not statistically significant predictors of call rate ($p = 0.11$; $p = 0.14$, respectively); however, circulating levels of CORT positively predicted call rate ($p = 0.02$; Fig. 5B). Similarly, the multiple regression model significantly predicted vocal effort ($F_{3,42} = 6.13$, $p = 0.001$; *adj*. $r^2 = 0.26$; Table 1). Circulating levels of DHT and T were not statistically significant predictors of vocal effort ($p = 0.13$; $p = 0.58$, respectively); however, circulating levels of CORT negatively predicted vocal effort ($p = 0.003$; Fig. 5C).

The multiple regression model significantly predicted the low-frequency component of male advertisement calls ($F_{3,39} = 4.56$, $p = 0.008$; *adj*. $r^2 = 0.20$; Table 1). Circulating levels of DHT and T were not statistically significant predictors of the low dominant carrier frequency ($p = 0.96$; $p = 0.55$, respectively); however, circulating levels of CORT positively predicted low-frequency ($p = 0.001$; Fig. 5D). Conversely, the multiple regression model did not significantly

I also ran a simple linear regression with CORT against the measured vocal parameters without data obtained from satellite males to explore how inclusion/exclusion of satellite data affected the association between CORT and vocal parameters. When solely calling host males were included in the analysis, CORT was not correlated with low frequency, call duration, call rate, or vocal effort ($p > 0.07$ for all correlations; Fig. 5A, B, C, & D). These results contrast with results obtained if satellites are included (Fig. 5) and corroborate previous findings that CORT among calling males is in such a narrow range that the relationship between CORT and acoustic parameters is undetectable (see Chapter two).

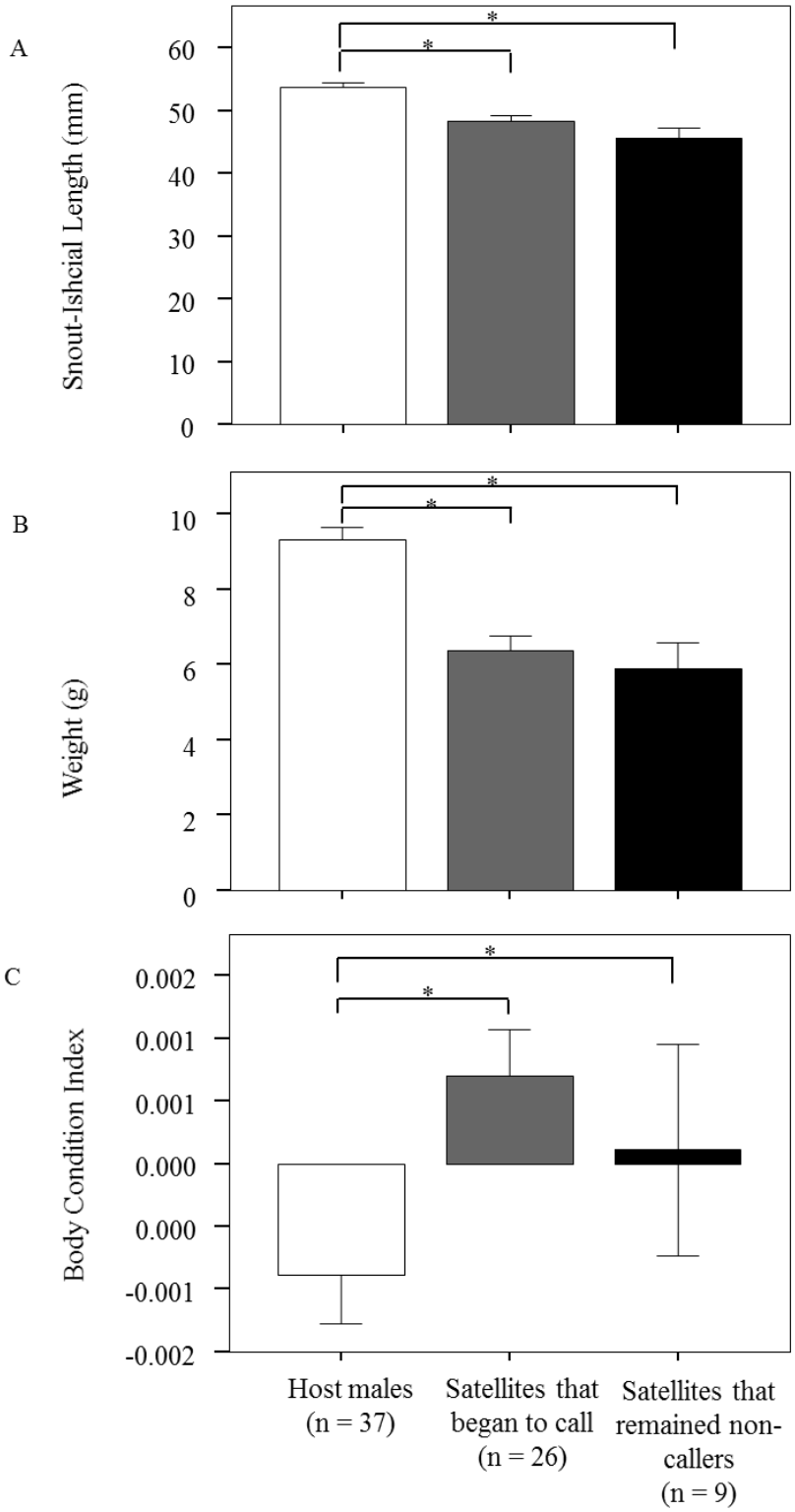

Figure 3. Bar graphs depicting the average (A) body size, (B) weight, and (C) condition of host and satellite males that did and did not call after host removal. Bar with asterisk indicates significant differences in size, weight and condition. Whiskers indicate ±1 SE.

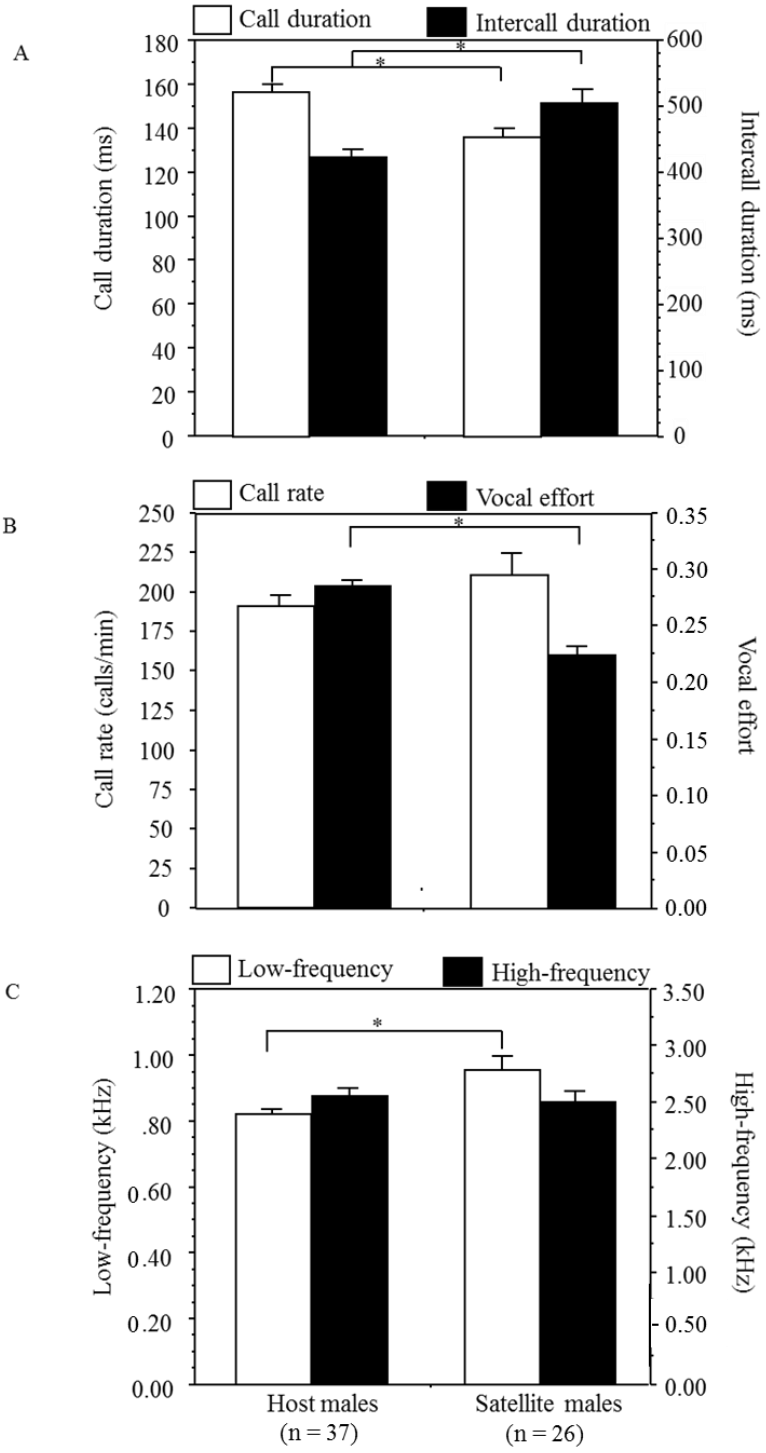

Figure 4. (A) Call and intercall duration, (B) call rate and vocal effort, and (C) low and high dominant carrier frequencies in host males and satellite males that called subsequent to host removal. Bar with asterisk indicates significant differences in vocal parameters. Whiskers indicate ±1 SE.

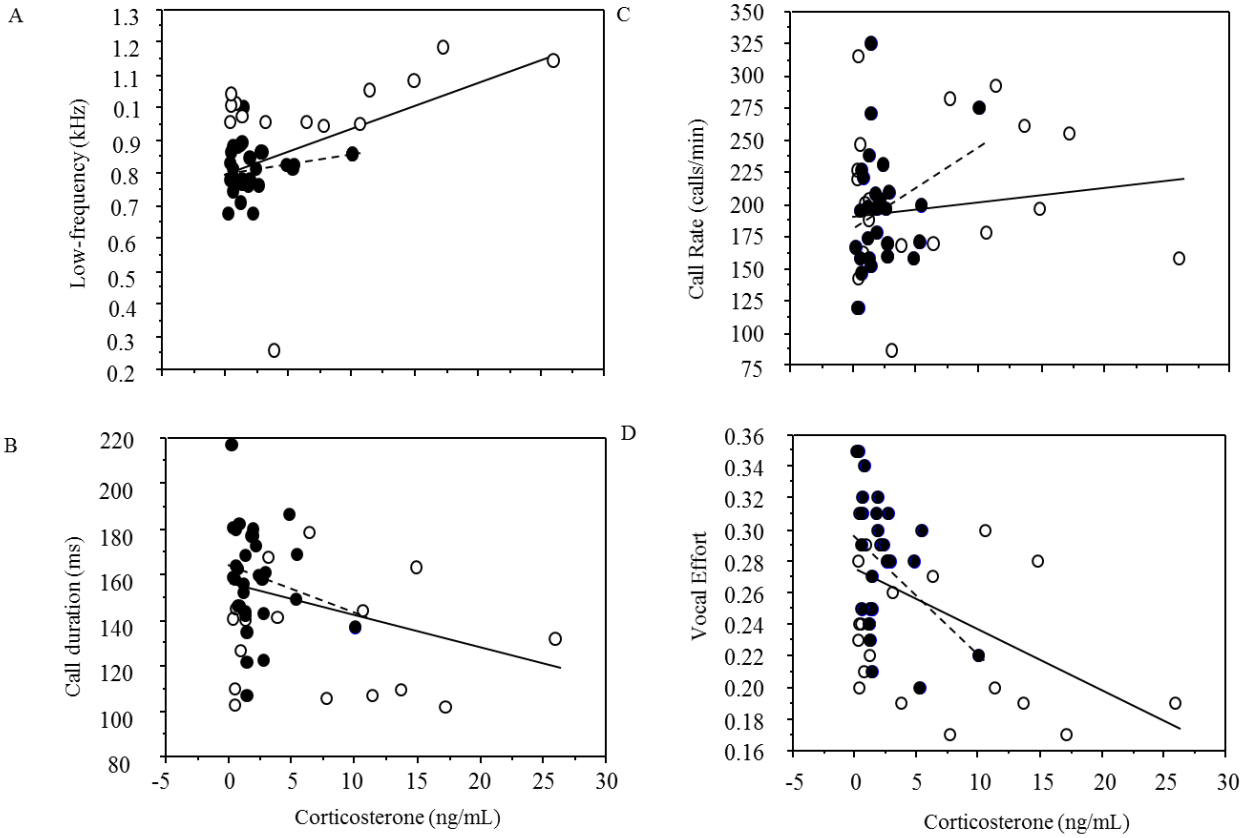

Figure 5. Scatterplots showing the correlation between circulating levels of corticosterone (CORT) and (A) low-frequency, (B) call duration, (C) call rate, and (B) vocal effort. The stippled line is the regression trend for host males only (solid circle); the solid line is the regression trend for both hosts and satellites (open circles).

Table 1. Results from a multiple regression analysis assessing the association between circulating hormone levels and acoustic parameters.

	Unstandardized Coefficients		Standardized Coefficients		
Parameters	**B**	**SE**	**Beta**	**t**	***p***
Call duration (ms)	0.47[a]	0.34	0.28	1.37	0.18
	-0.13[b]	0.61	-0.04	-0.22	0.83
	-1.41[c]	0.70	-0.29	-2.02	**0.05**
Intercall duration (ms)	0.05[a]	1.27	0.008	0.04	0.97
	-2.73[b]	2.25	-0.25	-1.21	0.23
	4.74[c]	2.61	0.27	1.82	0.08
Call Rate (calls/min)	-1.08[a]	0.65	-0.32	-1.65	0.11
	1.72[b]	1.16	0.29	1.49	0.14
	3.17[c]	1.34	0.33	2.36	**0.02**
Vocal Effort	0.001[a]	0.001	0.28	1.53	0.13
	0.001[b]	0.001	0.10	0.56	0.58
	-0.004[c]	0.001	-0.42	-3.16	**0.003**
Low-frequency (kHz)	$-9.8e^{-005}$[a]	0.002	-0.01	-0.05	0.96
	-0.002[b]	0.003	-0.12	-0.60	0.55
	0.02[c]	0.004	0.51	3.62	**0.001**
High-frequency (kHz)	0.008[a]	0.004	0.41	1.88	0.07
	-0.007[b]	0.007	-0.20	-0.92	0.36
	0.008[c]	0.009	0.14	0.87	0.39

B is the unstandardized coefficients, with their associated standard error (SE). Beta is the standardized coefficient.
[a]Coefficient for predictor variable DHT.
[b]Coefficient for predictor variable T.
[c]Coefficient for predictor variable CORT.
Bold indicates significance, alpha at 0.05.

Discussion

The results indicate that circulating androgen level predicts whether a satellite male vocalizes or continues to adopt non-calling satellite behavior after removal of the associated calling host male. For example, satellite males that continued to exhibit non-calling behavior

after host removal possessed lower circulating levels of DHT relative to both host males and satellites that began to call. However, satellites that continued to adopt non-calling satellite behavior and satellite males that began to call after removal of the calling host male did not differ in circulating CORT levels. Rather, satellite males that began to call possessed significantly higher circulating levels of CORT relative to calling host males. These results are consistent with my prediction that satellite males with elevated CORT level call upon removal of the calling host male, but that males with low androgens do not call after host removal. The high levels of circulating CORT in satellite males that began to call further support the context-dependency of CORT-mediated effects on calling behavior. For example, experimentally elevated CORT increases the probability of satellite behavior during social interactions with rival males (see Chapter 1).

Aggressive signals produced during male-male agonistic interactions can elicit elevations in circulating levels of CORT (Leary, 2014) which can decrease male vocal attractiveness (see Chapter 1). For example, experimentally elevated CORT results in a decrease in call duration and vocal effort (see Chapter 1). Consistent with these findings, CORT was negatively correlated with call duration and vocal effort when both satellites and callers were included in the analysis. In contrast, no such relationship was detected when using data from calling males only. This appears to stem from the fact that circulating CORT varies minimally across calling males (mean = 2.23 ng/ml ± 2.09; range = 0.2 – 10.1 ng/ml; also see Chapter 2), but that range becomes much broader when satellite males are included (mean = 5.65 ng/ml ± 6.89; range = 0.2 – 26.0 ng/ml). These findings suggest that high CORT level is a powerful determinant of mating tactic expression and can dramatically affect male courtship signals.

Males that are vocally unattractive relative to neighboring conspecifics are often expected to adopt satellite behavior (e.g., concepts of the relative attractiveness hypothesis; Waltz, 1982; Arak, 1988; see Chapter 2). Consistent with this hypothesis, males that adopt satellite behavior when challenged by a conspecific are generally vocally unattractive and produce calls of short duration and with low overall vocal effort (see Chapter 2). The negative effects of CORT on vocal attractiveness suggest that competing males use acoustic signals in an armament capacity to compromise vocal attractiveness of rival males (see Chapter 1). In such a scenario, it may be beneficial for the subordinate male to adopt satellite behavior to increase mating opportunities (see McCauley et al., 2000). While dominant calling males have a higher probability of mate acquisition, satellite males do occasionally acquire mates (Perrill et al., 1978; Miyamoto and Cane, 1980; Perrill et al., 1982; Arak, 1988; Perrill and Magier, 1988; Haddad, 1991; Bourne, 1992). For example, satellite hourglass treefrogs (*Hyla ebraccata*) have a 38% mating success rate compared to calling males (Miyamoto and Cane, 1980). In *H. cinerea*, 13 of 30 satellite males (43%) successfully intercepted females attracted to the host male's vocalizations (Perrill et al., 1982). In contrast, without a calling host to attract females to the calling territory, it may no longer be beneficial to adopt non-calling behavior; yet, my results indicate that 26% of satellite male *H. cinerea* did not call after host removal.

Calling in anurans is known to be androgen-dependent (Solis and Penna, 1987) which can explain the absence of vocalization in satellite males with low androgen levels after host removal. However, whether low androgens in satellite males are a consequence of negative effects of elevated CORT on androgen production is unclear. For instance, experimentally elevated CORT can elicit adoption of satellite behavior independently of changes in androgen levels (see Chapter 1). There is also no evidence that circulating levels of androgens are

associated with relative vocal attractiveness (see also Chapter 2). Rather than low androgens causing satellite behavior, these results suggest that low androgens may be a consequence of satellite tactic expression. For instance, in addition to stimulating CORT production, aggressive signals produced by host males may also elicit a decrease in circulating levels of androgens (see Leary, 2014) thereby suppressing the calling behavior of competitively inferior males.

The results also indicate that satellite males are smaller and in poorer body condition relative to host males. The energetic constraints of being a small male in poor body condition may regulate the magnitude of glucocorticoid secretion in response to stressors (Dunlap and Wingfield, 1995; Kitaysky et al., 1999; Jessop et al., 2002; Moore and Jessop, 2003; Leary, 2014). For example, small male *H. cinerea* show a greater increase in CORT production (and decrease in DHT levels) in response to broadcast advertisement calls (Leary, 2014). This potentially explains the consistent observation that satellites are generally smaller than host males (Forester and Lykens, 1986; Eggert and Guyetant, 2003; Leary et al., 2015; Castellano et al., 2009; Brepson et al., 2012). Body size is considered a major determinant of competitive ability (Parker, 1974; Smith and Parker, 1976; Smith, 1982) and male anurans assess the body size of rival males using dominant carrier frequencies of acoustic signals (Robertson, 1986; Wagner, 1992) which may contribute to male tactic decisions.

Smalls males appear to be at a major disadvantage as a result of high glucocorticoid responsiveness to agonistic interactions with rival males (Leary, 2014). Body size is the most robust predictor of mating tactic expression and can influence tactic expression independently of variation in circulating hormone levels (see Chapter 2). It is possible that high circulating levels of CORT are also a consequence of males adopting satellite behavior following assessment of body size against rival males. For instance, mutual assessment of body size can enable a

competitively inferior male to choose to adopt satellite behavior rather than engaging in agonistic contests that could result in injuries (Berec and Bajgar, 2011). Following adoption of satellite behavior, new social circumstances potentially elicit changes in circulating levels of CORT and androgens (see Chapter 2).

Oliveira et al. (2008) proposed that differences in circulating hormone levels in males practicing different mating tactics may be a cause or consequence of mating tactic expression. In other words, hormone levels can alter the probability that a behavior is expressed, but expressing a particular behavior can also give rise to variation in hormone levels (Oliveira et al., 2002; Oliveira, 2004; 2005 Adkins-Regan, 2005). The bidirectionality of hormone behavior relationships is apparent in dominant-subordinate relationships of species that establish dominance hierarchies. In teleost fish, for example, dominant behavior can give rise to high circulating levels of androgens (Oliveira et al., 2002). In many non-cooperative breeders, subordinance is generally predicted by low baseline levels of glucocorticoids (Coe et al., 1979; Rohwer and Wingfield, 1981; Schwabl et al., 1988; Virgin and Sapolsky, 1997) and, in some species, subordinate males also show high glucocorticoid responsiveness to stressors (*reviewed* by Gilmour et al., 2005). This relationship between subordinance rank and circulating levels of glucocorticoids gave rise to the "subordinance is stressful hypothesis" (*reviewed* by Creel, 2001).

Essentially, there is a cost to being a subordinate male that may reflect endocrine state. In anurans, one potential cost to subordinate status is high circulating levels of CORT, stimulated by acoustic contests, that can decrease vocal attractiveness. Under such conditions, the satellite mating tactic may offer the highest chance of reproductive success. Satellite tactic expression, however, may be costly if exposure to male calls causes reductions in circulating androgens because such effects suppress calling behavior even in the absence of dominant calling males.

Such effects could have a major impact on reproductive success because male mating success is often positively correlated with time spent calling (*reviewed* by Gerhardt and Huber, 2002). The results suggest that satellite males may be subject to such costs if vocal interactions with dominant males while adopting satellite behavior causes a reduction in circulating androgen levels.

Conclusion

Species in which males employ alternative mating tactics to increase mating opportunities provide a valuable system to study the role of circulating hormones in intrasexual competition (*reviewed* by Miles et al., 2007). Male *H. cinerea* produce acoustic armaments that stimulate the production of glucocorticoids and decrease androgens in signal receivers (Leary, 2014). I previously showed that elevations in CORT can decrease vocal attractiveness and increase the probability that males will adopt satellite behavior (see Chapter 1). Furthermore, the effects of elevated CORT on mating tactic expression is context-dependent and most prominent during simulated territorial intrusions (see Chapter 1). Consistent with the context-dependent effect of CORT on mating tactic expression, previous satellite-caller manipulation experiments demonstrate that a proportion of satellite males will begin to vocalize after removal of the host male (Perrill et al., 1982; Arak, 1988; Humfeld, 2008). Here, I demonstrate that non-calling behavior of satellite males after removal of the host is predicted by low androgen levels. My results suggest that competing males can decrease rival male fitness by eliciting a decrease in androgens.

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LIST OF APPENDICES

Appendix A. Correlation matrix showing relationships among dependent variables.

Measure	1	2	3	4	5	6	7	8	9	10	11	12	13
(1) DHT	1												
(2) T	.75**	1											
(3) CORT	.25*	.11	1										
(4) Call duration	.08	.13	-.04	1									
(5) Intercall duration	.07	-.01	-.04	.45**	1								
(6) Call rate	.06	.04	.36**	-.32**	-.18	1							
(7) Vocal effort	.01	.13	-.02	.49**	-.52**	-.19	1						
(8) Low-frequency	-.03	-.09	.01	-.07	.06	-.14	-.13	1					
(9) High-frequency	-.04	-.12	.19	-.21	-.29*	.04	.07	.15	1				
(10) SIL	-.07	.13	-.08	.30*	.11	.01	.19	-.37**	-.33**	1			
(11) Weight	.11	.28*	-.26*	.29*	.09	-.03	.18	-.37**	-.44**	.81**	1		
(12) Condition	.32**	.28*	-.32*	.09	-.03	-.09	.06	-.09	-.25*	-.05	.54**	1	
(13) Glucose	.01	-.08	.02	.18	.04	.004	.07	-.19	.04	.27*	.12	-.21	1

*Correlation is significant at the 0.05 level.
**Correlation is significant at the 0.01 level.

VITAE

Sarah Crocker-Buta

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EDUCATION

Master of Science, Biological Sciences, University of Mississippi, December 2016
Advisor: Dr. Christopher Leary

Bachelor of Science, Biological Sciences, University of Alabama, 2013

EMPLOYMENT

University of Mississippi — Oxford, Mississippi
Teaching Assistant, BISC 331 — August 2016 – December 2016

University of Mississippi — Oxford, Mississippi
Research Assistant, Dr. Elaine Day — June 2016 – July 2016

University of Mississippi — Oxford, Mississippi
Research Assistant, Dr. William Resetarits — January 2016 - May 2016

University of Mississippi — Oxford, Mississippi
Teaching Assistant, BISC 331 — August 2015 – December 2016

University of Mississippi — Oxford, Mississippi
Teaching Assistant, BISC 163 — January 2015 – May 2015

University of Mississippi — Oxford, Mississippi
Teaching Assistant, BISC 331 — August 2014 - December 2014

University of Mississippi — Oxford, Mississippi
Teaching Assistant, BISC 163 — January 2014 - May 2014

University of Mississippi — Oxford Mississippi
Teaching Assistant, BISC 161 — August 2013 -December 2013

University of Alabama — Tuscaloosa, Alabama
Student IT assistant, virus removal from student computers — Sept. 2010 - May 2013

RESEARCH EXPERIENCE

Neuroendocrinology Lab, University of Mississippi, Oxford, MS.
PI: Prof. Chris Leary, University of Mississippi — August 2013 - Present

Evolutionary and Integrative Physiology Lab, University of Alabama, Tuscaloosa, AL.
PI: Prof. Stephen Secor, University of Alabama. — January 2012 – May 2013

Evolutionary Ecology and Biogeography Lab, University of Alabama. Tuscaloosa, AL.
PI: Prof. Lesslie Rissler, University of Alabama. — August 2010 – December 2010

AWARDS AND FUNDING

2016 University of Mississippi Field Station Graduate Student Research Grant ($500), Oxford, MS

2015 3MT competition, 1st place master's division, University of Mississippi, Oxford, MS

2015 Summer Graduate Student Research Assistantship, University of Mississippi ($2500), Oxford, MS

2015 Graduate Student Council Research Grant, University of Mississippi ($1000), Oxford, MS

2015 University of Mississippi Field Station Graduate Student Research Grant ($500), Oxford, MS

2014 Summer Graduate Student Research Assistantship, University of Mississippi ($2500), Oxford, MS

2013 Randall Outstanding Undergraduate Research Award, University of Alabama ($250), Tuscaloosa, Al

ACADEMIC APPOINTMENTS

Teaching Assistant, Comparative Anatomy of the Vertebrates Laboratory, University of Mississippi, Fall 2015
Prof. Chris Leary

Teaching Assistant, Biological Sciences II Laboratory, University of Mississippi, Spring 2015
Prof. Gail Stratton

Teaching Assistant, Comparative Anatomy of the Vertebrates Laboratory, University of Mississippi, Fall 2014
Prof. Chris Leary.

Teaching Assistant, Biological Sciences II Laboratory, University of Mississippi, Spring 2014
Prof. Gail Stratton.

Teaching Assistant, Biological Sciences I Laboratory, University of Mississippi, Fall 2013. Prof. Gail Stratton.

RELATED EXPERIENCE

Day camp outreach, Grades 5 - 6
University of Mississippi Field Station June 2015

Day camp outreach, Grades 5 - 6
University of Mississippi Field Station June 2014

CONFERENCE PRESENTATIONS

Crocker-Buta, S.P and Leary, C.J. 2016. Social and endocrine factors influence mating behavior in male treefrogs. Poster presentation at the 2016 Annual meeting of The Society for Integrative and Comparative Biology, Portland, OR.

Crocker-Buta, S.P and Leary, C.J (2015) Extrinsic and intrinsic determinants of mating behavior in male green treefrogs, *Hyla cinerea*. Poster presentation at the 2015 Annual meeting of the Society for the Study of Amphibians and Reptiles, Lawrence, KS.

Crocker-Buta, S.P and Leary, C.J (2015) Variation in the responses of male green treefrogs to vocal playbacks: Does relative attractiveness or endocrine state predict mating tactic expression? Poster presentation at the 2015 Annual meeting of The Society for Integrative and Comparative Biology, West Palm Beach, FL

Crocker-Buta, S.P and Secor, S.M (2013) Determinants and repeatability of specific dynamic action for the corn snake *Pantherophis guttatus*. Oral presentation at the University of Alabama 2013 Annual Undergraduate Research and Creative Activity Conference, Tuscaloosa, AL.

Crocker-Buta, S.P and Secor, S.M (2013) Determinants and repeatability of specific dynamic action for the corn snake *Pantherophis guttatus*. Poster presented at the University of Alabama 4th Annual Frontiers in Biology Colloquium, Tuscaloosa, AL.

Crocker-Buta, S.P and Secor, S.M (2013) Determinants and repeatability of specific dynamic action for the corn snake *Pantherophis guttatus*. Poster presented at the 2013 Annual meeting of The Society for Integrative and Comparative Biology, San Francisco, CA.

Crocker-Buta, S.P., Nectman, S., and Secor, S.M. (2012) Impact on meal size and body size on the cost of digestion for the corn snake. Poster presented at the University of Alabama 2012 Annual Undergraduate Research and Creative Activity Conference, Tuscaloosa, AL.

PUBLICATIONS

Crocker-Buta, S.P., Secor, S.M. 2014 Determinants and repeatability of the specific dynamic response of the corn snake, *Pantherophis guttatus*. Comparative Biochemistry and Physiology Part A. 169, 60-69.

PROFESSIONAL ASSOCIATIONS

American Society of Ichthyologists and Herpetologists, 2014 - present
Animal Behavior Society, 2013 – present
Society for Integrative and Comparative Biology, 2013 – present
Society for the Study of Amphibians and Reptiles, 2012 – present